Cellular Responses to Congenital Disorders of Glycosylation

Dissertation

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Summary

Glycosylation is a frequent protein modification in all kingdoms of life. In humans, 50% of all proteins are glycosylated. The structural diversity that is created by glycosylation is tremendous. This high diversity allows glycans to participate in many processes in the human body. Inborn defects of glycosylation in humans lead to diseases known as Congenital Disorders of Glycosylation (CDG). These disorders present developmental defects especially of the nervous system, coagulation disorders, tissue fibrosis, hormonal disturbance and more. Today, more than 40 forms of CDG are known with mutations in glycosyltransferases, in enzymes of the substrate synthesis, in carbohydrate-nucleotide transporters, and others. These mutations are the direct causes of the glycosylation defects, but the molecular mechanisms relating abnormal glycosylation to cellular and organ phenotypes are often unknown.

In this thesis, I report the characterization of a novel form of CDG and the investigation of cellular responses to underglycosylation in CDG. In the first part of the thesis, a mutation in the *COG5* gene of an untyped CDG patient is described and directly associated with the disease phenotype. For the first time it was shown that a rather mild form of CDG is caused by a vesicular transport defect. This description is important for pediatricians to consider a COG defect in similar cases. In the second part, the role of the ER glucosyltransferases ALG6, ALG8 and ALG10 in the glycosylation, maturation and secretion of marker glycoproteins was investigated. To this end, the ER glucosyltransferase genes were inactivated in HeLa cells by shRNA-mediated knockdown. It was shown that the knockdown approach worked. However, there were no significant differences of the investigated cellular parameters between the knockdown cells. In the third part, the cellular responses to abnormal glycosylation were investigated on a global level. The transcriptomes of different CDG and healthy control fibroblasts were compared, which revealed a strong induction of extracellular matrix gene expression in CDG fibroblasts. This response has been confirmed at the protein level and it was shown that the insulin-like growth factor binding protein (IGFBP) 5 could play an important role in the induction of extracellular matrix gene expression.

Overall, the work presented in this thesis contributes to the understanding of the cellular responses to abnormal glycosylation and therefore to the understanding of CDG and their

symptoms. The description of a new form of CDG is clinically important and the involvement of IGFBP5 in tissue fibrosis in CDG can point to a potential target for therapy.

Zusammenfassung

Die Glykosylierung ist eine häufige Proteinmodifikation, die in allen Lebewesen vorkommt. Im Menschen ist ungefähr die Hälfte aller Proteine glykosyliert. Durch Glykosylierung entsteht eine enorme Vielfalt an Strukturen, die im menschlichen Körper an ganz unterschiedlichen biologischen Prozessen teilnehmen. Eine fehlerhafte Glykosylierung führt im Menschen zu Krankheiten mit dem Namen „Angeborene Glykosylierungskrankheiten“ (CDG). Diese Krankheiten sind charakterisiert durch Entwicklungsstörungen vor allem des Nervensystems, durch Blutgerinnungsstörungen, Gewebsfibrosen, hormonelle Störungen und andere Symptome. Heute kennt man mehr als 40 Formen von CDG, die durch Mutationen in Glykosyltransferasen, in Enzymen der Substratsynthese, in Zuckernukleotidtransportern und anderen Proteinen ausgelöst sind. Diese Mutationen sind direkt verantwortlich für die fehlerhafte Glykosylierung. Die molekularen Mechanismen jedoch, welche durch die abnormale Glykosylierung zu den zellulären und organellen Phänotypen führen, sind oft nicht bekannt.

In dieser Doktorarbeit charakterisierte ich eine neue Form von CDG, und ich untersuchte die zellulären Antworten auf Unterglykosylierung in CDG. Im ersten Teil wird eine Mutation im *COG5* Gen in einem nicht-charakterisierten CDG Patienten beschrieben und direkt in Zusammenhang mit dem Krankheitsbild gebracht. Zum ersten Mal wurde gezeigt, dass eine recht milde Form von CDG durch einen Defekt im vesikulären Transport ausgelöst werden kann. Diese Beschreibung ist wichtig für Kinderärzte, damit sie bei ähnlichen Fällen einen COG-Defekt in Betracht ziehen. Im zweiten Teil wurde die Rolle der ER Glukosyltransferasen ALG6, ALG8 und ALG10 in der Glykosylierung und der Reifung und Sekretion von Markerglykoproteinen untersucht. Um dies zu erreichen wurden die Gene der ER Glukosyltransferasen mittels shRNA in HeLa Zellen inaktiviert. Es wurde gezeigt, dass der methodische Ansatz funktionierte, dass aber bei den gemessenen, zellulären Parametern keine signifikanten Unterschiede existierten. Im dritten Teil wurden die zellulären Antworten auf abnormale Glykosylierung auf einer umfassenden Ebene angeschaut. Die Transkriptome verschiedener CDG- und Kontrollfibroblasten wurden verglichen, was eine starke Induktion der Expression von extrazellulären Matrix Genen in CDG Fibroblasten aufzeigte. Dieses Resultat wurde auf der Proteinebene bestätigt und es wurde auch gezeigt, dass das Insulin-

ähnliche Wachstumsfaktor-Bindungsprotein (IGFBP) 5 eine wichtige Rolle spielen könnte bei der Induktion der Expression von extrazellulären Matrix Genen.

Als Ganzes trägt diese Doktorarbeit zum Verständnis von den zellulären Antworten auf abnormale Glykosylierung und damit zum Verständnis von CDG und deren Symptome bei. Die Beschreibung einer neuen Form von CDG ist klinisch wichtig und die Beteiligung von IGFBP5 an der Gewebsfibrose in CDG kann auf einen potentiellen Angriffspunkt für eine Therapie hindeuten.

Introduction to glycobiology

Glycobiology is the research field investigating the synthesis, structure and function of carbohydrates that modify proteins and lipids. Proteins are translated from a template, the mRNA, which has been transcribed from DNA. This linear, template-driven process of protein synthesis produces a pool of polypeptides. These peptides can then be modified posttranslationally, adding additional levels of complexity. Chemical groups like phosphates are added or removed from proteins to modulate their function (1). Lipids are transferred to proteins to anchor them in membranes (2). The polypeptide ubiquitin is attached to proteins to mark them for degradation (3). And the most frequent protein modification, glycosylation, has a huge variety of roles that will be described in this introduction. Glycosylation is the enzymatic attachment of carbohydrates to proteins or lipids by glycosyltransferases and is found in all kingdoms of life, including some viruses (4). Thus, glycosylation is a highly conserved and important protein modification from single-cell to multicellular organisms. The significance of glycosylation is further highlighted by the fact that approximately 50% of all human proteins are modified by glycans and around 1 to 2% of the human genome encodes for proteins involved in the process of glycosylation (5). In contrast to other protein modifications, glycosylation reactions create a large diversity of structures. In mammals, nine different carbohydrate monosaccharides are used for glycan synthesis. Glycan synthesis generally involves sequential addition of monosaccharides to a growing glycan chain. Glycan synthesis is initiated by attachment of a monosaccharide to different chemical groups of amino acids or lipids. The type of chemical group modified, on a specific peptide or lipid substrate, by a specific monosaccharide provides a first level of diversity and complexity to the process of glycosylation. A second level of complexity derives from a diversity of chemical linkages between monosaccharides of carbohydrate chains. Further complexity arises from the length of the carbohydrate chain and the presence or absence of branches in the chain. A final level of complexity arises from heterogeneity at sites of glycosylation, where specific sites in a protein or lipid can be modified by different glycan structures or no glycan at all depending on the cell type or the stage of development. The level of diversity is such that even identical substrates produced in a single cell can acquire different glycan compositions. This high degree of heterogeneity and complexity implies a broad range of functions for glycans and requires a high level of control of glycosylation reactions (6).

The structure of glycans

In mammalian cells, the nine different monosaccharides glucose (Glc), galactose (Gal), mannose (Man), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), sialic acid (Sia), xylose (Xyl), and glucuronic acid (GlcA) are used as building blocks for glycans (7) (shown in figure 1). Further modifications of these monosaccharides such as epimerization of GlcA to iduronic acid (IdoA) and sulfation of Gal, GlcNAc, GalNAc, GlcA and IdoA can occur after the incorporation of the monosaccharides into a glycan chain (8, 9). Sia can be hydroxylated already in the cytosol before incorporation into the glycan chain in some mammals, however not in humans since the responsible hydroxylase is not active. Furthermore, Sia can be modified by *O*-acetylation and *O*-methylation after incorporation into the glycan chain in the Golgi apparatus (10, 11).

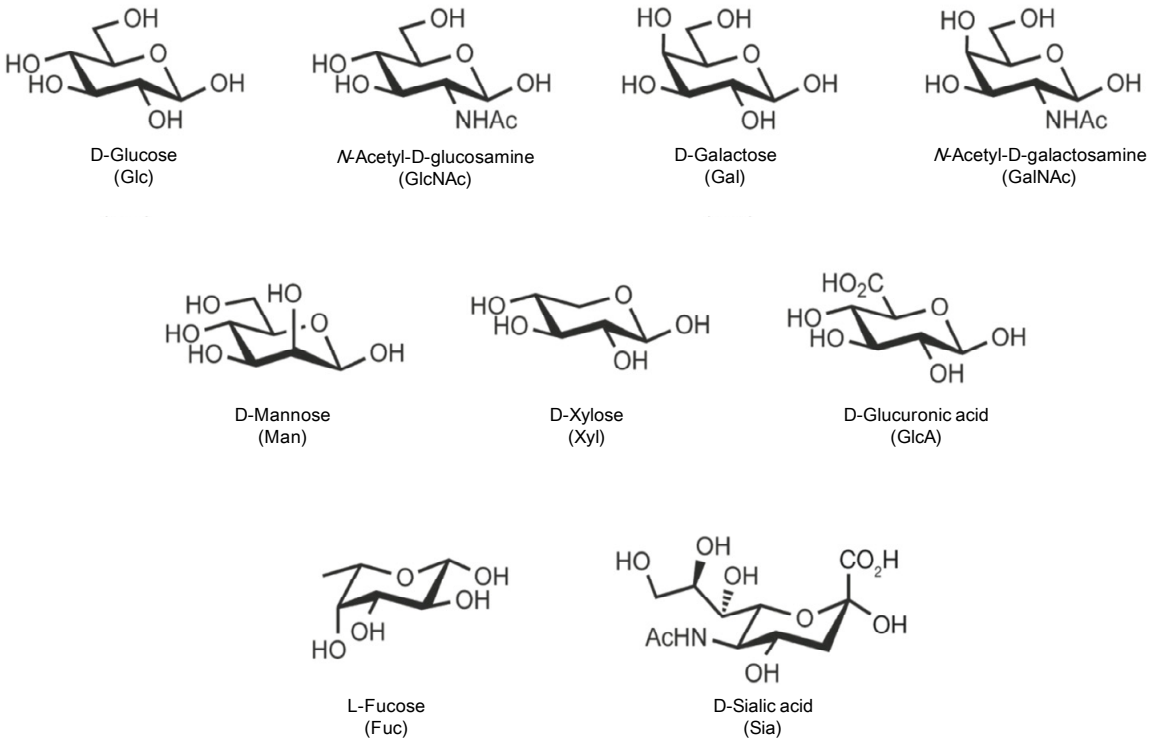

Figure 1 Chemical structure of the nine carbohydrates used in mammalian glycoconjugates. Adapted from (6).

In mammals, seven different classes of glycans are known. As shown in figure 2, each class originates from a common core structure. The core structure of most of the classes can further be elongated and branched. However, these additional modifications are specific to the cell producing them and are diverse. The most common glycoconjugate in eukaryotes is referred to as an *N*-glycan because the carbohydrate chain is linked to the substrate through

a nitrogen in an amino acid side chain (4). The *N*-glycan core structure is composed of $GlcNAc_2$ Man_3 attached to Asn in the consensus sequence Asn-Xaa-Ser/Thr. Xaa can be any amino acid except Pro. For *O*-glycans that are linked to a substrate through the oxygen of a hydroxyl group of Ser or Thr, no universal consensus for the acceptor sequence exists. One reason is that the transfer of the first carbohydrate of the most common O-glycan, the mucin-type O-glycan, is catalyzed by a big family of GalNAc-transferases that have different acceptor specificities (12, 13). Furthermore, the core structure of *O*-glycans is more diverse than in *N*-glycans because different carbohydrates can be attached to Ser or Thr. Besides GalNAc also Man, Glc and Fuc can modify Ser or Thr. In the particular case of collagen glycosylation, Gal is conjugated to hydroxylysine (14). The so called *C*-glycans modify a C-atom of Trp in the consensus sequence Trp-Xaa-Xaa-Trp with the first Trp being the acceptor (15). Glycosaminoglycans (GAG) are long carbohydrate chains with repeating disaccharide units mainly found in proteoglycans (16). The core structure of heparan sulfates and chondroitin sulfates is composed of Xyl followed by two Gal on Ser (17). Besides amino acids, lipids can also be modified by glycans. In glycosphingolipids Gal or Glc is attached to ceramide (18). Four carbohydrates in the glycosylphosphatidylinositol (GPI) anchor link a luminal protein to phosphatidylinositol in the membrane (19). The seventh class of glycosylation is cytoplasmic/nuclear glycosylation. In this class, GlcNAc is linked to the hydroxyl group of Ser or Thr and is not further modified or elongated. In contrast to all the previously described glycosylation classes, cytoplasmic/nuclear glycosylation is a dynamic modification, which is analogous to protein phosphorylation. Indeed, often the same hydroxyl groups are phosphorylated or GlcNAc-ylated and these two modifications seem to compete with each other on the same substrate (20, 21).

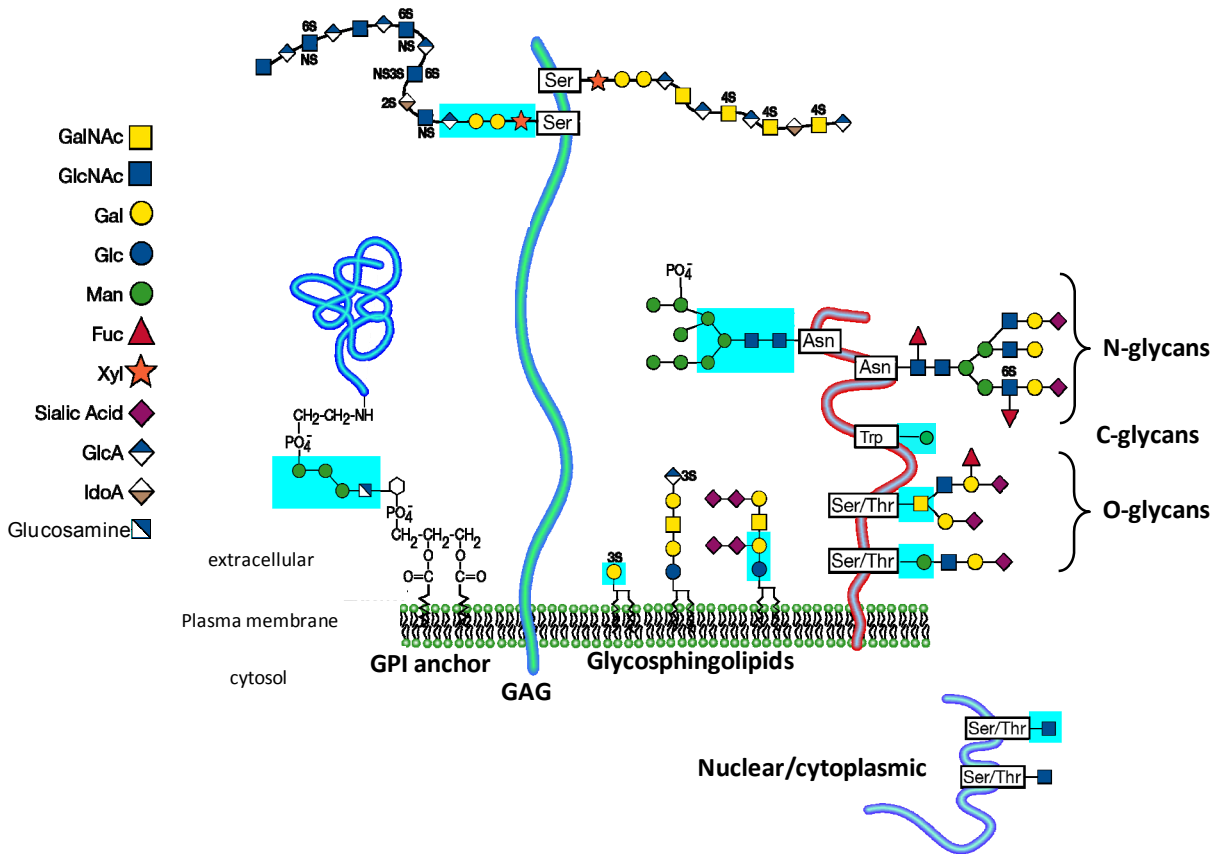

Figure 2 The seven classes of mammalian glycoconjugates given by their respective core structures.

The core structures are highlighted in cyan. The carbohydrates elongating the core structures are examples and can be different from glycan to glycan. Adapted from (6).

The synthesis of glycans

Glycan biosynthesis mainly takes place at the endoplasmic reticulum (ER) membrane and in the Golgi apparatus. The exception is cytoplasmic/nuclear glycosylation. Key components of glycosylation reactions are the glycosyltransferases, which catalyze the formation of a glycosidic bond. Most Golgi glycosyltransferases are type II transmembrane proteins with a short cytosolic tail and a large luminal catalytic domain, whereas many ER glycosyltransferases are multi-transmembrane spanning proteins (22). With few exceptions, glycosyltransferases are specific for acceptor substrate, donor substrate, and linkage. Therefore, they can be named by the donor substrate that they utilise and the carbohydrate linkage they produce. Enzymes that catalyze the same glycosidic bond are grouped in families.

In figure 3, a schematic overview of a glycosyltransferase reaction is shown. The donor substrates for glycosyltransferases are nucleotide or dolichol activated carbohydrates. Nucleotide activated carbohydrates are synthesised in the cytosol, except CMP-Sia, which is synthesised in the nucleus (23) and UDP-Xyl, which is generated from UDP-GlcA in the ER and Golgi (24). Dolichol activated carbohydrates are synthesised on the cytosolic side of the ER on the lipid dolichol. For glycosylation reactions in the ER and Golgi lumen, the donors have to be transported across the membrane. The hydrophilic nucleotide activated carbohydrates are transported via antiporters into these organelles. These antiporters exchange one molecule of nucleotide-P-P-carbohydrate with one molecule of nucleotide-P, which is the by-product of the glycosyltransferase reaction (25). Dolichol activated carbohydrates have to be flipped across the ER membrane to expose the carbohydrate to the lumen. However, the flipping process is not yet understood.

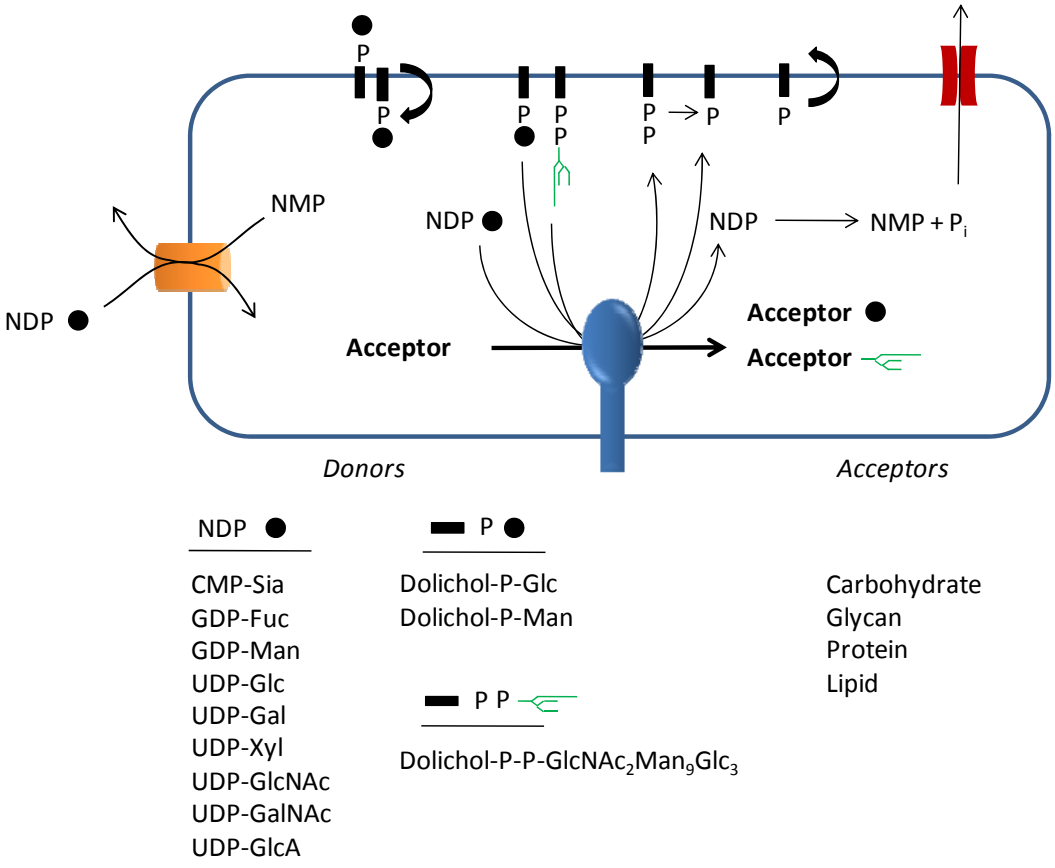

Figure 3 Schematic presentation of the glycosylation reaction in the secretory pathway.

Many glycosyltransferases inside the ER use dolichol activated carbohydrates as substrate. Golgi glycosyltransferases use nucleotide activated carbohydrates. The acceptor can be an amino acid, a lipid, or a carbohydrate. The reaction product dolichol-P-P of lipid-linked oligosaccharide (LLO) synthesis has to be dephosphorylated and recycled back to the cytoplasmic side of the membrane. The reaction product of Golgi glycosyltransferases, nucleotide-P-P, has to be dephosphorylated and nucleotide-P is transported out of the lumen in exchange for a carbohydrate-nucleotide-P-P. The inorganic phosphate exits the lumen via an unknown transporter.

While the diversity of glycan structures is huge, the carbohydrate sequence of a glycan is not random. The acceptor specificity of glycosyltransferases limits the number of possible glycan structures. Furthermore, glycosyltransferases are localised to distinct portions of the secretory pathway in the ER and the various Golgi stacks. Therefore, they act sequentially on the proteins that are transported through the secretory pathway. The expression and distribution of glycosyltransferases has to be tightly controlled to produce the correct glycan structure(s) on distinct substrates.

The synthesis of N*-glycans*

The synthesis of *N*-glycans is presented in figures 4 and 6. The process starts on and in the ER by the assembly of a precursor glycan on dolichol-P, thereby forming a lipid-linked oligosaccharide (LLO) (figure 4).

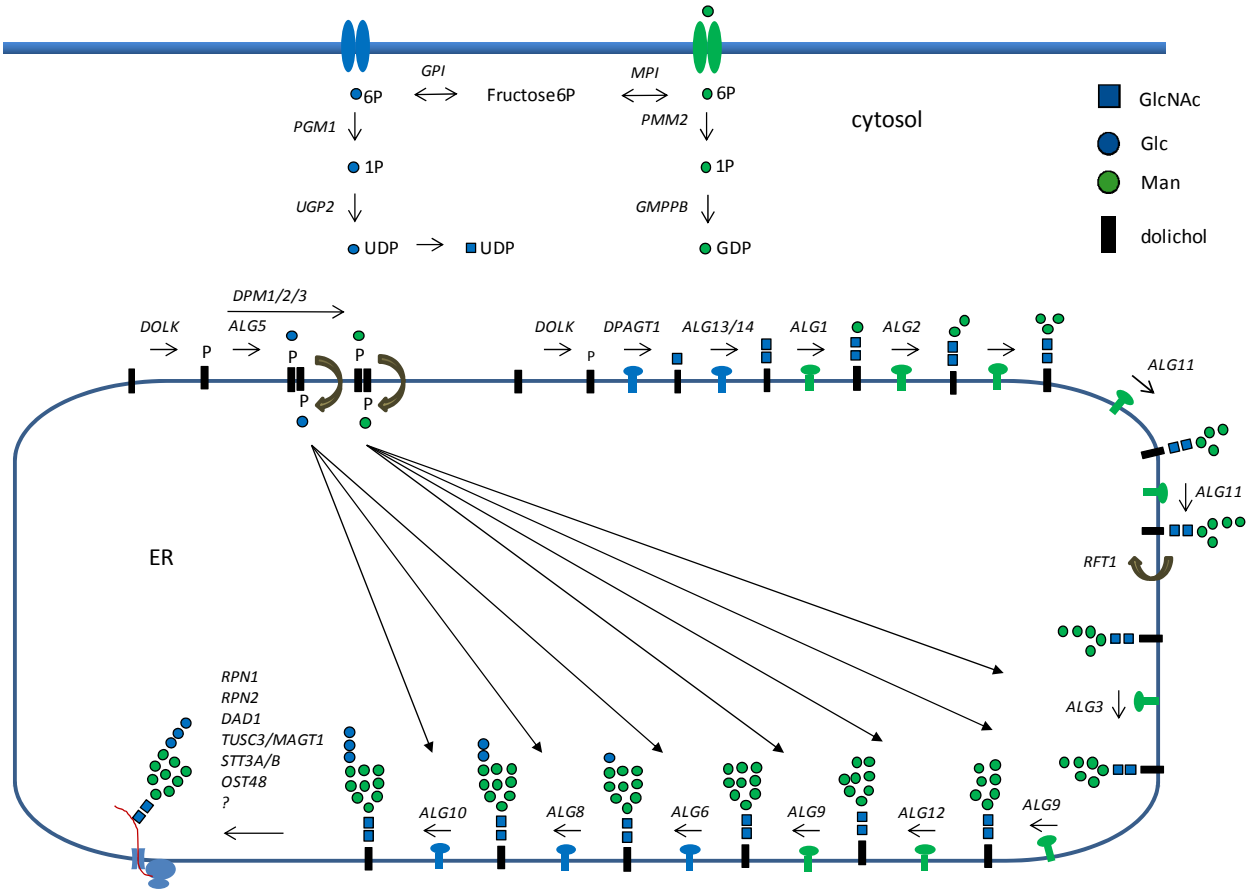

Figure 4 The *N*-glycosylation pathway in the ER.

The synthesis of the *N*-glycan starts at the ER membrane by the assembly of the lipid-linked oligosaccharide (LLO). The incomplete LLO is translocated across the membrane by a flippase. The complete LLO is transferred by the multi-subunit protein complex oligosaccharyltransferase (OST) to the newly synthesised protein. The gene symbols are given in italics according to http://www.genenames.org/.

In humans, dolichol lipid is synthesised via the mevalonate pathway resulting in dolichol-P product with 18 -21 condensed isoprene units (26). Dolichol-P acts as the carrier of the LLO as well as the carrier of Man and Glc, which are used as activated carbohydrates for the elongation reaction inside the ER (27-29). Up to the structure Dolichol-P-P-$GlcNAc_2Man_5$ the synthesis takes place on the cytosolic side of the ER. The Dolichol-P-P-$GlcNAc_2Man_5$ precursor is flipped across the membrane by a not yet completely understood process. However, the protein RFT1 seems to play an important role (30). The LLO is elongated inside the ER lumen to the structure $GlcNAc_2Man_9Glc_3$ as shown in figure 5. The oligosaccharyltransferase (OST), a multi-subunit protein complex, transfers the complete LLO from dolichol-P-P to *N*-glycosylation consensus sites on nascent polypeptides that enter through the SEC61 translocon (31). This reaction results in the so called *N*-linked oligosaccharide (NLO). The transfer is normally accomplished co-translationally by the catalytic OST subunit STT3A (32).

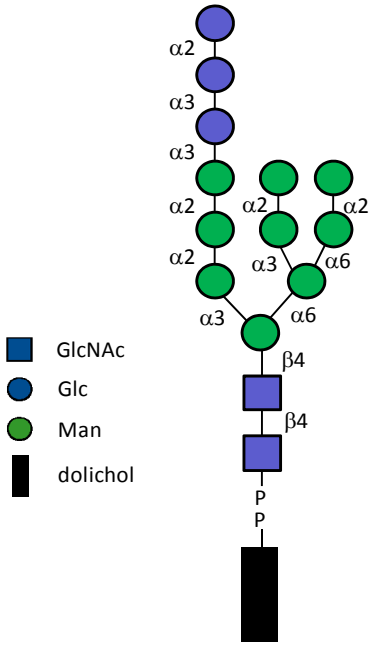

Figure 5 The structure of the complete lipid-linked oligosaccharide (LLO).

Carrier of the LLO is dolichol-P (black rectangle) in the ER membrane.

LLO assembly, like all glycan formation, is a sequential process because glycosyltransferases are acceptor specific and modify only the structure produced by the preceding glycosyltransferase. Deficiencies in glycosyltransferases cause the accumulation of incomplete LLOs. Since the OST prefers complete LLOs, defects in a glycosyltransferase leads

to unoccupied *N*-glycosylation sites on secreted proteins. Underglycosylation of proteins is harmful and leads to diseases known as congenital disorders of glycosylation (CDG).

Recently, it was reported that a second isoform of STT3 (*STT3B*) is able to transfer LLOs post-translationally to the protein as long as the protein is not folded (33). Substrate specificity of the STT3B subunit is less stringent and incomplete LLOs can be transferred as well. This mechanism probably ensures that essential glycosylation sites are occupied and suggests that site occupancy is more important than the correct glycan structure at a specific site.

The transfer of the glycan by the OST results in an NLO on the protein and a dolichol-P-P in the membrane. Dolichol-P-P is de-phosphorylated and flipped back to the cytosolic side of the ER for reuse (34). The NLO is trimmed in the ER and immediately after arrival in the Golgi apparatus. Once the trimming process is complete the NLO is elongated to the mature glycan shown in figure 6.

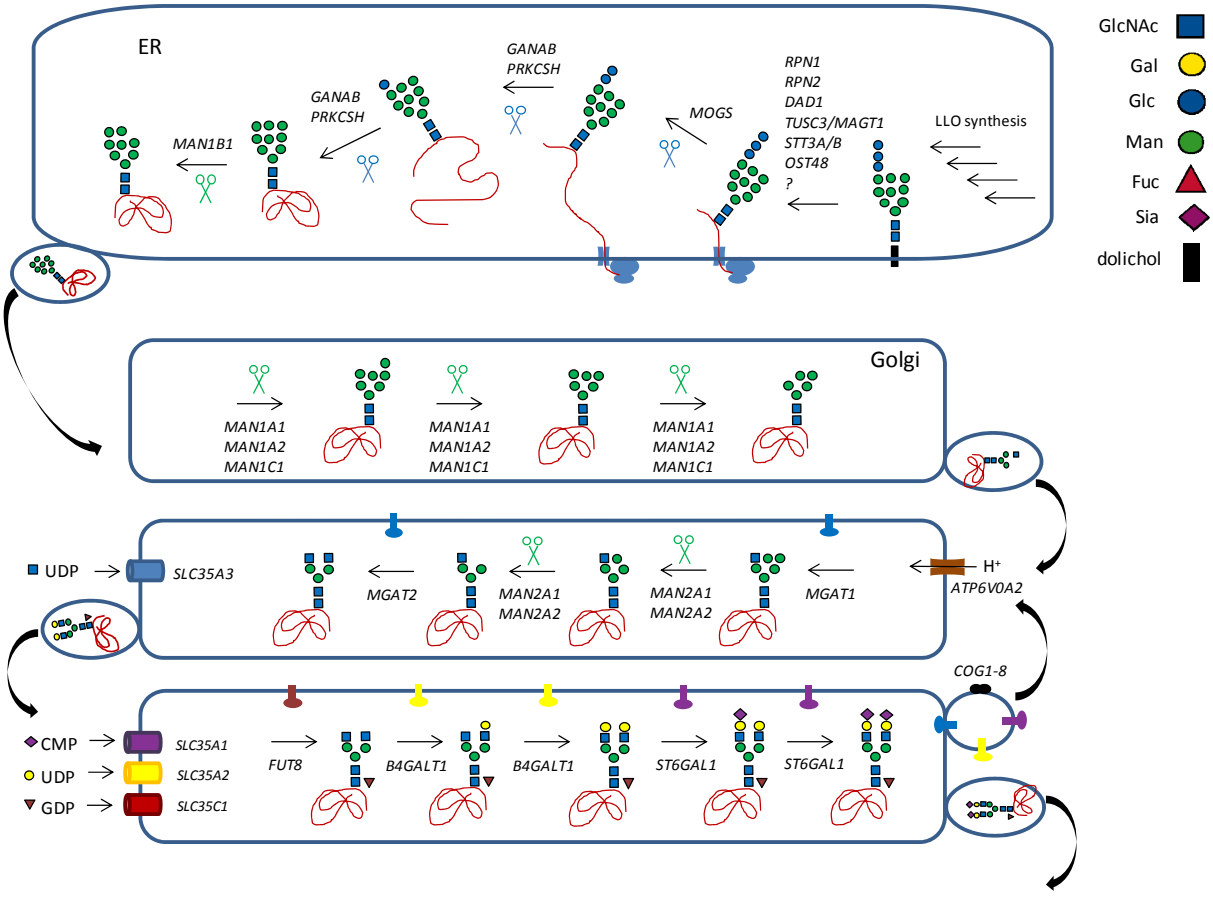

Figure 6 Trimming and elongation of the *N*-linked oligosaccharide (NLO) in the ER and the Golgi apparatus.

After transfer of the glycan from dolichol-P-P to Asn, the NLO is cleaved by glucosidases and mannosidases in the ER and the cis Golgi. Subsequent elongation of the glycan happens in the medial and trans Golgi. Antiporters are necessary for the donor import into the Golgi. The COG complex is involved in the retrograde transport of Golgi glycosyltransferases. The import of protons into the Golgi is necessary for the acidification of the Golgi cisternae. Folded and glycosylated mature proteins are exported from the trans Golgi network to their destination. The gene symbols are given in italics according to http://www.genenames.org/.

Two distinct glucosidases cleave the three Glc monosaccharides from the NLO in the ER (35). During this cleavage process the protein is folded. The correctly folded proteins are transported to the Golgi apparatus by vesicular transport. Cleavage of the four α1,2-linked Man monosaccharides begins during transport to the Golgi and is completed once there (36). GlcNAc is added to the α1,3-linked Man (37, 38) and the two outermost Man of the α1,6-branch are cleaved (39). The remaining α1,6-linked Man is elongated by another GlcNAc (38, 40). The structure of the glycan is completed by glycosyltransferases in the Golgi apparatus with specificities for Gal, Sia, and Fuc among others (27, 41). The final structure of the NLO can contain as many as four branches created by GlcNAc-transferases3/4/5 (41, 42) (not shown on figure 6). Depending on the degree of processing and elongation of the *N*-glycan in the Golgi apparatus the *N*-glycans are classified as high mannose, hybrid or complex type (shown in figure 7). High mannose type glycans only carry Man as terminal carbohydrates. In hybrid type glycans, one branch of the NLO is processed and elongated in the Golgi apparatus. The other branch remains unprocessed. Complex type glycans are completely processed in the Golgi apparatus and have all the branches elongated with carbohydrates other than Man (6, 27).

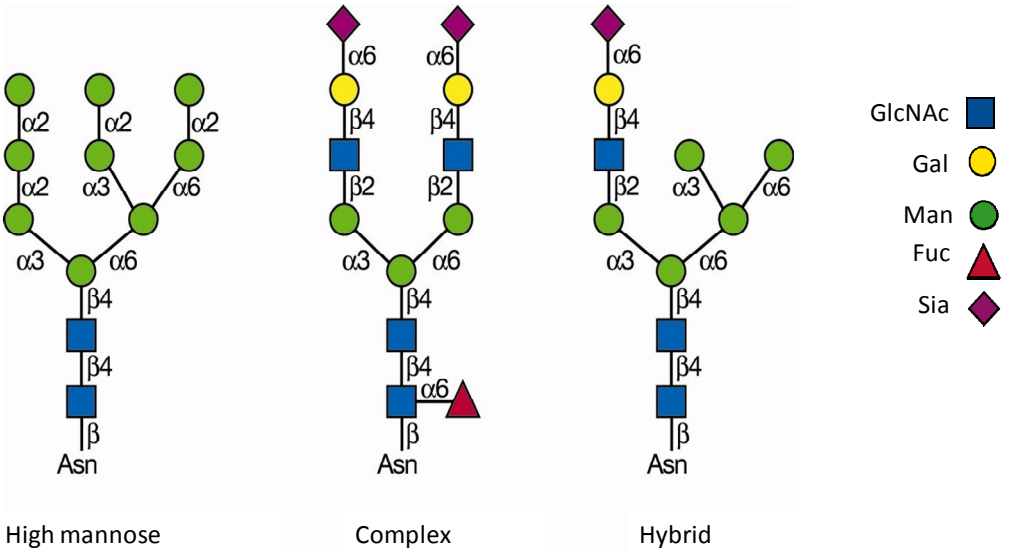

Figure 7 The three classes of *N*-linked glycans.

Depending on the degree of processing of the *N*-glycan, and the structural complexity of the branches, the *N*-glycan is classified as high mannose, hybrid or complex. Taken from (6).

The functions of glycans

The functions of glycans can be investigated by different approaches. *N*-glycosylation can be completely inhibited by the antibiotic tunicamycin. Tunicamycin is a nucleoside analogue and inhibits the initial GlcNAc-P transferase of LLO synthesis. Furthermore, several of the glycosidases involved in processing *N*-linked glycans can be inhibited by chemical agents in cell culture as well as in organisms. Mutations or knockouts of genes of the glycosylation machinery or mutations of glycosylation sites help to elucidate the role of glycans in mice. Naturally occurring mutations in CDG patients give insight into the complex action of glycans in human beings. However, the diversity of glycans is tremendous and so are their functions. Therefore, for many glycans, a function has not been identified. Nevertheless, some principles have emerged. Some glycans have structural and modulatory properties, while others are ligands both intra- and extracellularly (43).

The structural and modulatory properties of glycans can be seen in the extracellular matrix. The extracellular matrix is composed of proteoglycans and fibrous proteins such as collagens forming scaffolds. The GAG chains of proteoglycans contribute to the strength and stability of the extracellular matrix. GAG chains are hydrophilic and negatively charged and therefore can store water molecules and produce gels such as in cartilage (17, 44, 45). Similarly, in mucus, the *O*-glycan chains of mucin glycoproteins bind water and build up the viscous gels on the epithelium of the lungs and the intestinal and genital tracts, protecting the underlying epithelium from pathogens (46). The GAGs heparan and chondroitin sulfate not only retain water molecules but also bind cytokines and growth factors. They participate in the formation of gradients of cytokine and growth factors and are therefore important for developmental processes (47).

Glycans are also involved in binding reactions and often act as ligands recognized by proteins. The *O*-glycans of α-dystroglycan for example, participate in the binding of the dystrophin glycoprotein complex to extracellular matrix proteins like laminin. The dystrophin glycoprotein complex is the linker between the intracellular cytoskeleton and the extracellular matrix (48). Therefore, underglycosylation of α-dystroglycan causes severe muscular dystrophies (49).

Glycans can also be the receptors for pathogens. The influenza virus protein hemagglutinin binds to α2,3-Sia expressed in avian intestine or to α2,6-Sia expressed in the human

respiratory tract. It is the specificity for the glycan linkages in the receptor used by avian and human viruses that makes an influenza virus specific for the host. However, exchange of genetic material between viruses, and mutations in hemagglutinin can also change the host specificity allowing the species barrier to be crossed, contributing to the development of pandemics (50).

These examples show that glycans play a role in very diverse biological processes. Hence, it is not difficult to imagine that defects in glycosylation can lead to disease. Investigation of the functional role of glycans is therefore required in order to understand the underlying cause for several diseases, including the topic of this thesis, the CDG.

The functions of N-glycans

The *N*-glycan has two rather independent roles. On one hand, the *N*-glycan is important intracellularly where it supports the folding and secretion of newly synthesised proteins (51). On the other hand, the *N*-glycan is abundant extracellularly on the cell surface and on secreted proteins (52). There, it can protect the underlying polypeptide from degradation, it can be an interaction partner between cells or between the cell and the extracellular matrix, or it can be a regulator of protein activity.

The most prominent intracellular role of *N*-glycans is participation in quality control of protein folding. This role begins after the transfer of the carbohydrate moiety from the LLO to the nascent polypeptide in the ER. As shown in figure 8, the membrane-bound glucosidase1 cleaves the first Glc immediately after the transfer of the glycan to the Asn. The di-glucosylated glycan is substrate for the soluble glucosidase2 that cleaves the second Glc. The monoglucosylated form of the protein is able to bind to the lectins calnexin (membrane protein) or calreticulin (soluble protein). These two proteins are chaperones supporting the folding process (53). They recruit ERp57 to the unfolded protein. ERp57 is a protein disulfide isomerase that catalyzes the formation of disulfide bridges, especially in glycoproteins (53, 54). The binding of the glycoprotein to the chaperones is transient and glucosidase2 cleaves the last Glc. If the protein is correctly folded, it can be transferred to the Golgi apparatus. The unglucosylated, not yet completely folded glycoprotein is recognized by the glycoprotein glucosyltransferase, which acts as a protein folding sensor. How this protein distinguishes misfolded or unfolded from correctly folded is not yet fully understood. Probably hydrophobic stretches of misfolded proteins are recognized and lead to the reglucosylation

of the misfolded protein (55). The resulting monoglucosylated protein is again a substrate for calnexin and calreticulin and enters another folding cycle.

Terminally misfolded proteins are exported from the ER into the cytosol. There, the glycoprotein is deglycosylated, ubiquitinated and degraded by the 26S proteasome. This process is called ER-associated protein degradation (ERAD) (56). Cleavage of α1,2-linked Man by ER-mannosidase1 and maybe by other ER degradation enhancing α-mannosidase-like (EDEM) proteins is thought to be the signal for degradation. ER mannosidase1 acts slowly and seems to be a clock that gives the signal for degradation after several rounds of folding (57). However, there are also recent publications claiming that the Man residues are not important for ERAD (58). The exact mechanism for recognition of misfolded proteins and their translocation still has to be explored.

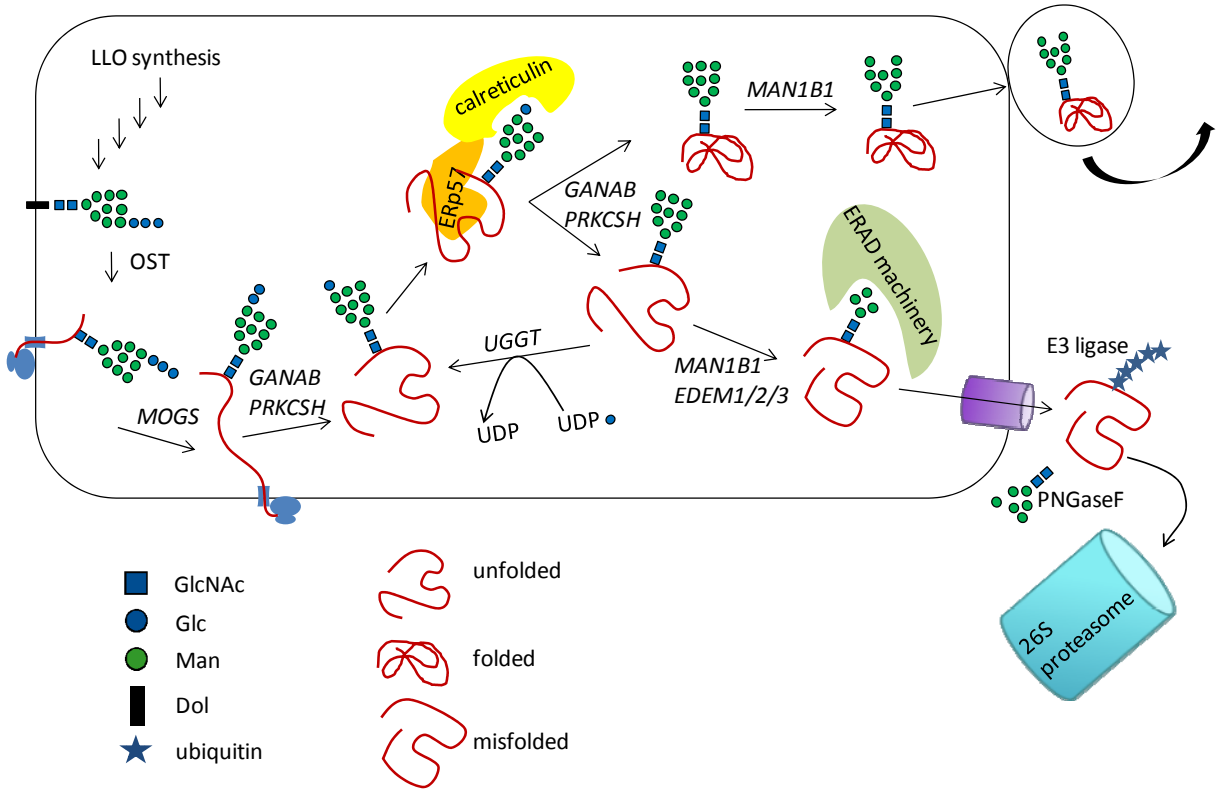

Figure 8 The quality control mechanism of glycoprotein folding in the ER of mammalian cells.

After the transfer of the glycan structure from the LLO to the nascent polypeptide chain by the OST, the glycan is cleaved by glucosidase1 and glucosidase2. The monoglucosylated form is the substrate for the folding machinery calnexin/calreticulin/ERp57. Glucosidase2 removes the last Glc and the protein can be secreted to the Golgi if correctly folded. If the protein is misfolded, glycoprotein glucosyltransferase reglucosylates the protein and binding to the folding machinery is repeated. Finally, misfolded proteins are demannosylated and exported by the ER associated protein degradation (ERAD) machinery from the ER into the cytosol. In the cytosol the protein is deglycosylated, ubiquitinated and degraded by the proteasome. The gene symbols are given in italics according to http://www.genenames.org/.

Another intracellular role for *N*-glycans is targeting of lysosomal proteins to the correct compartment (59, 60). Lysosomal proteins carry *N*-glycans that are modified in the Golgi apparatus by GlcNAc-P transferase and GlcNAc-1-P diesterase creating a Man-6-P tag (61). This Man-6-P tag marks lysosomal proteins and is recognized by the Man-6-P receptors that transport the bound proteins to the endosomes and finally to the lysosomes. The receptor-ligand complex dissociates at the lower pH of the lysosomes and the receptor is recycled (61). Mutations in the GlcNAc-1-P transferase lead to a low abundance of proteases in the lysosomes, and therefore cause a lysosomal storage disease known as mucolipidosis (62, 63).

The extracellular roles of *N*-glycans are less well defined than the intracellular ones because a huge variety of structures exist and no ubiquitous structure-function-related function has been described. The following paragraphs specify examples demonstrating the diversity and range of functions.

The hormone erythropoietin (EPO) is essential for the development of erythrocytes. It is a glycoprotein with three *N*-glycans and one *O*-glycan (64, 65). The recombinant production of the hormone is of high value for the treatment of anaemia. The *N*-glycans of EPO are of the complex type and are terminally sialylated. It was recognized that sialylation is important for bioactivity (66). Desialylated EPO is active *in vitro* but not *in vivo*. This has been attributed to desialylated EPO being cleared faster from the circulation (67-69). Thus, recombinant human EPO is produced in Chinese hamster ovary (CHO) cells which possess a glycosylation machinery similar to human cells. The introduction of two additional, artificial glycosylation sites to EPO was observed to further increase the half-life of EPO in the circulation (70).

Correct *N*-glycosylation can also be important for receptor activity. Core fucosylation of the TGFβ1 receptor is necessary for correct signal transduction through this receptor. Knockout mice of the α1,6-Fuc transferase present severe growth retardation, lung destruction, and 70% die within the first days after birth (71).

Moreover, *N*-glycans can be directly bound by proteins, called lectins, and therefore act as ligands. Selectins for example, are carbohydrate binding proteins in a family called C-type lectins (72). Selectins are subdivided into three classes depending on the cell type they are expressed in. L-selectin is predominantly found on leukocytes, P-selectin on activated platelets and endothelial cells and E-selectin on activated endothelial cells. Selectins

participate in the interaction between leukocytes and the endothelium during rolling and extravasation of leukocytes into lymphatic organs (73). However, they also play a role in leukocyte-tumor cell interaction during metastasis (74, 75). All selectins bind to so called sialyl Lewis^x carbohydrate structures shown in figure 9 (76-78). This structure is found on *O*- and *N*-glycans of specific proteins. The sialyl Lewis^x structure can be further modified by sulfation. Sulfated sialyl Lewis^x is specifically bound by L-selectin (9). The importance of the sialyl Lewis^x structure is highlighted by a disease called leukocyte adhesion deficiency II (79). Patients have a mutation in the gene encoding the GDP-fuc transporter of the Golgi apparatus. They are unable to fucosylate the Lewis^x structure. Therefore, they present leukocyte adhesion deficiency and thus immunodeficiency (80).

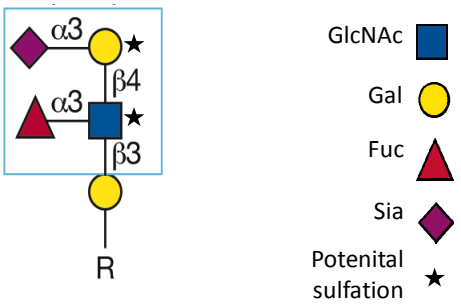

Figure 9 The sialyl lewis^x structure.

The sialyl lewis^x motif is indicated by a cyan box. The R can be either an *N*-or an *O*-glycan. Adapted from (6).

Terminal carbohydrates of the band3 protein of erythrocytes define the human ABO blood group system. However, these terminal structures are also found on *N*- and *O*-glycans of other proteins and on glycolipids. The A allele of the *ABO* locus codes for an α1,3-GalNAc transferase. The B allele, which differs only in four amino acids, codes for an α1,3-Gal transferase. The O allele codes for an inactive form of the enzyme. Therefore, individuals present different ABO blood groups depending on the expressed alleles (shown in figure 10) (81). The blood group does not have an influence on the health condition of an individual. However, antibodies are raised against A epitopes in B group individuals and vice versa. If blood of two different blood groups is mixed, the erythrocytes agglutinate. Therefore, blood transfusion is only possible between the same blood groups or from a O individual to any other.

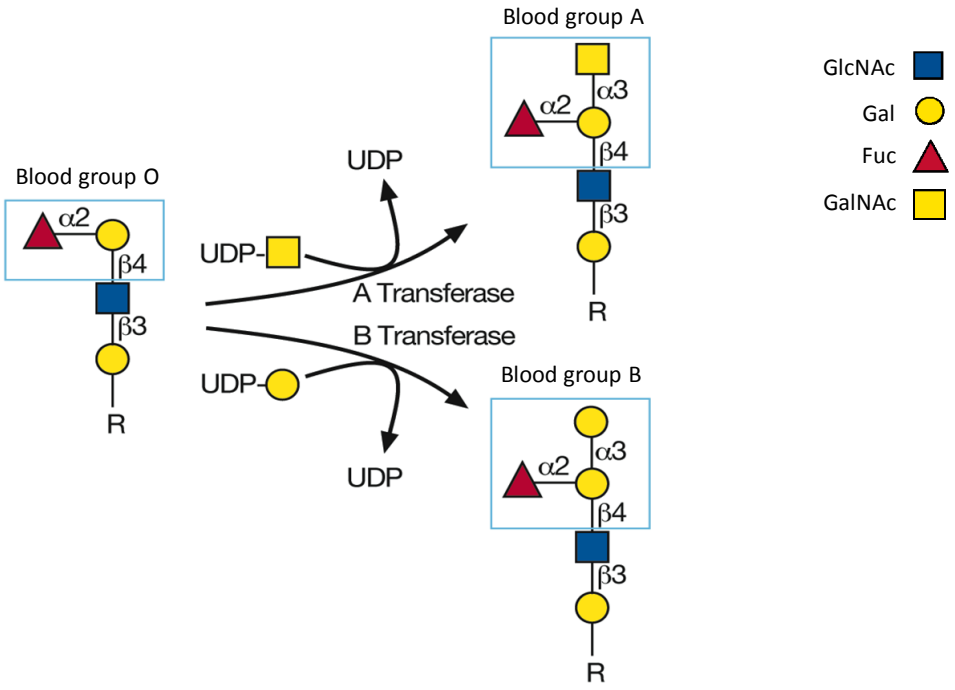

Figure 10 The human ABO blood group.

The antigens of the ABO blood group are surrounded by a cyan box. R can be either an *N*- or an *O*-glycan or a glycolipid. Depending on the allele present in the ABO locus, the A or the B transferase is expressed. In the case of the O blood group an inactive form of the enzyme is expressed. Adapted from (6).

Taken together, the spectrum of *N*-glycan functions is huge and far from being completely understood. *N*-glycans have pivotal roles in biological processes and abrogation of *N*-glycosylation is not compatible with life. Knockout of enzymes of the LLO synthesis pathway and of the Golgi localised β1,2-GlcNAc transferase1 are lethal (82-85). Therefore, hybrid type *N*-glycans are the minimum prerequisite for life. Terminal carbohydrates of complex type *N*-glycans are often involved in binding reactions. Disruption of terminal carbohydrates eliminates the binding capability of these *N*-glycans and therefore often causes severe disease.

Diseases of glycosylation

Defects in the glycosylation machinery or alterations in glycosylation patterns can cause disease in human beings. Genetic defects in the glycosylation machinery are named congenital disorders of glycosylation (CDG) (86, 87). Today, mutations in more than 40 genes are identified as causing CDG. The number of known patients is rather small as only about 1000 cases have been described worldwide. However, CDG is probably under-diagnosed. The broad spectrum of symptoms makes it difficult to recognize CDG in clinics. Furthermore, about 20% of the affected individuals die in the first two years of life and are therefore often not diagnosed.

CDG defects are divided into four different classes depending on the glycosylation pathway affected. These are *N*-glycosylation, *O*-glycosylation, lipid glycosylation and multiple glycosylation disorders. The individual diseases are named after the affected gene (88). Currently, 45 genes are known that lead to CDG if one of the genes is mutated. They are listed in table 1. Until recently, the defects were classified into two groups, CDG type I and CDG type II. CDG type I corresponded to all diseases with defects of *N*-glycosylation site occupancy. CDG type II referred to the diseases with defects of *N*-glycan processing and elongation. This classification was recently changed because disorders with deficient *O*- and lipid- glycosylation or with defects in multiple glycosylation pathways were discovered.

N-glycosylation defects

Disease name	**Defective protein**	**OMIM**
PMM2-CDG (*CDG-Ia*)	Phosphomannomutase 2	212065
MPI-CDG(*CDG-Ib*)	Phosphomannose isomerase	602579
ALG6-CDG (*CDG-Ic*)	Dol-P-Glc:Man_9-$GlcNAc_2$-P-P-Dol glucosyltransferase (glucosyltransferase 1)	603147
ALG3-CDG (*CDG-Id*)	Dol-P-Man:Man_5-$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase 6)	601110
ALG12-CDG (*CDG-Ig*)	Dol-P-Man:Man_7-$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase 8)	607143
ALG8-CDG (*CDG-Ih*)	Dol-P-Glc:Glc_1-Man_9-$GlcNAc_2$-P-P-Dol glucosyltransferase (glucosyltransferase 2)	608104
ALG2-CDG (*CDG-Ii*)	GDP-Man:Man_1-$GlcNAc_2$-P-P-Dol	607906

	mannosyltransferase (mannosyltransferase 2)	
DPAGT1-CDG (*CDG-Ij*)	UDP-GlcNAc:Dol-P-GlcNAc-P transferase	608093
ALG1-CDG (*CDG-Ik*)	GDP-Man:$GlcNAc_2$-P-P-Dol mannosyltransferase (mannosyltransferase 1)	608540
ALG9-CDG (*CDG-Il*)	Dol-P-Man:Man_6-and Man_8-$GlcNA_2$-P-P-Dol mannosyltransferase (mannosyltransferase 7-9)	608776
RFT1-CDG (*CDG-In*)	Flippase of $Man_5GlcNAc_2$-PP-Dol	612015
MGAT2-CDG (*CDG-IIa*)	N-acetylglucosaminyltransferase 2	212066
MOGS1-CDG (*CDG-IIb*)	Glucosidase 1	606056
TUSC3-CDG (autosomal recessive nonsyndromic mental retardation)	Oligosaccharyltransferase subunit	611093
MAGT1-CDG (nonsyndromic x-linked mental retardation)	Oligosaccharyltransferase subunit	300176

O-glycosylation defects

Defective gene (disease)	**Defective protein**	**OMIM**
EXT1/EXT2-CDG (multiple cartilaginous exostoses)	Glucuronyltransferase/*N*-acetylglucosaminyltransferase	133700/ 133701
B4GALT7-CDG (Ehlers-Danlos syndrome, progeroid form)	β-1,4-galactosyltransferase 7	130070
GALNT3-CDG (familial tumoral calcinosis)	Polypeptide *N*-acetylgalactosaminyltransferase 3	211900
SLC35D1-CDG (Schneckenbecken dysplasia)	Solute carrier family 35 (UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter), member D1	269250
POMT1/POMT2-CDG (Walker-Warburg syndrome, congenital muscular dystrophy spectrum)	Protein-*O*-mannosyltransferase 1/protein-*O*-mannosyltransferase 2	236670
POMGNT1-CDG (Muscle-eye-brain disease, congenital muscular dystrophy spectrum)	Protein-*O*-mannose β-1,2-*N*-acetylglucosaminyltransferase	253280
FKTN -CDG (Fukuyama muscular dystrophy)	Fukutin	253800

FKRP-CDG (muscular dystrophy, limb-gridle, type 2I)	Fukutin-related protein	607155
LARGE-CDG (congenital muscular dystrophy, type 1D)	*N*-Acetylglucosaminyltransferase-like protein	608840
LFNG-CDG (spondylocostal dysostosis type 3)	*O*-Fucose-specific β-1,3-*N*-acetylglucosaminyltransferase	609813
B3GALTL-CDG (Peters plus syndrome)	*O*-Fucose-specific β-1,3-glucosyltransferase	261540

Glycolipid defects

Defective gene (disease)	**Defective protein**	**OMIM**
ST3GAL5-CDG (Amish infantile epilepsy)	Lactosylceramide α-2,3 sialyltransferase (GM3 synthase)	609056
PIGM-CDG (glycosylphosphatidylinositol deficiency)	Phosphatidylinositolglycan, class M	610293

Multiple glycosylation defects

Defective gene (disease)	**Defective protein**	**OMIM**
DPM1-CDG (CDG-Ie)	GDP-Man:Dol-P-mannosyltransferase subunit 1 (Dol-P-Man synthase, subunit 1)	608799
DPM3-CDG (CDG-Io)	GDP-Man:Dol-P-mannosyltransferase subunit 3 (Dol-P-Man synthase, subunit 3)	612937
MPDU1-CDG (CDG-If)	Man-P-Dol utilization 1	609180
B4GALT1-CDG (CDG-IId)	β-1,4-galactosyltransferase 1	607091
GNE-CDG (hereditary inclusion body myopathy)	UDP-GlcNAc epimerase/kinase	600737
SLC35A1-CDG (CDG-IIf)	CMP-sialic acid transporter	603585
SLC35C1-CDG (CDG-IIc) (Leukocyte adhesion deficiency, type II)	GDP-fucose transporter	266265
DOLK-CDG (*CDG-Im*)	Dolichol kinase	610768
COG7-CDG (*CDG-IIe*)	Subunit 7 of conserved oligomeric Golgi complex	608779
COG1-CDG (*CDG-IIg*)	Subunit 1 of conserved oligomeric Golgi complex	611209

COG8-CDG (*CDG-IIh*)	Subunit 8 of conserved oligomeric Golgi complex	611182
COG4-CDG (*CDG-IIj*)	Subunit 4 of conserved oligomeric Golgi complex	606976*
COG5-CDG	Subunit 5 of conserved oligomeric Golgi complex	606821*
ATP6V0A2-CDG (cutis laxa type II)	Subunit A2 of vesicular H^+-ATPase	219200
SEC23B-CDG (congenital dyserythropoietic anemia type II)	COPII component SEC23B	224100

Table 1 Known genetic defects causing CDG.

The gene names plus the common name of the disease is given in the first column. The protein name and the accession number for the OMIM database for the disease or the gene (*) are given in the second and third columns. Adapted from (88).

The routine clinical test to identify CDG patients with defects in *N*-glycosylation is isoelectric focusing (IEF) of serum transferrin (89). In figure 11 an example of an IEF of two different CDG patients and a control is shown. Transferrin carries two *N*-glycans with each *N*-glycan having two branches. Therefore, most transferrin carries four terminal Sia. Sia is negatively charged, which enables the separation of transferrin glycoforms with varying numbers of Sia in the electric field. Healthy control transferrin shows a strong band at the position of four charges because of complete sialylation. CDG patients with a deficiency in site occupancy show pronounced bands at the position of two and zero charges. In contrast, CDG patients with mutations in genes affecting *N*-glycan processing and elongation present pronounced bands at any position below four.

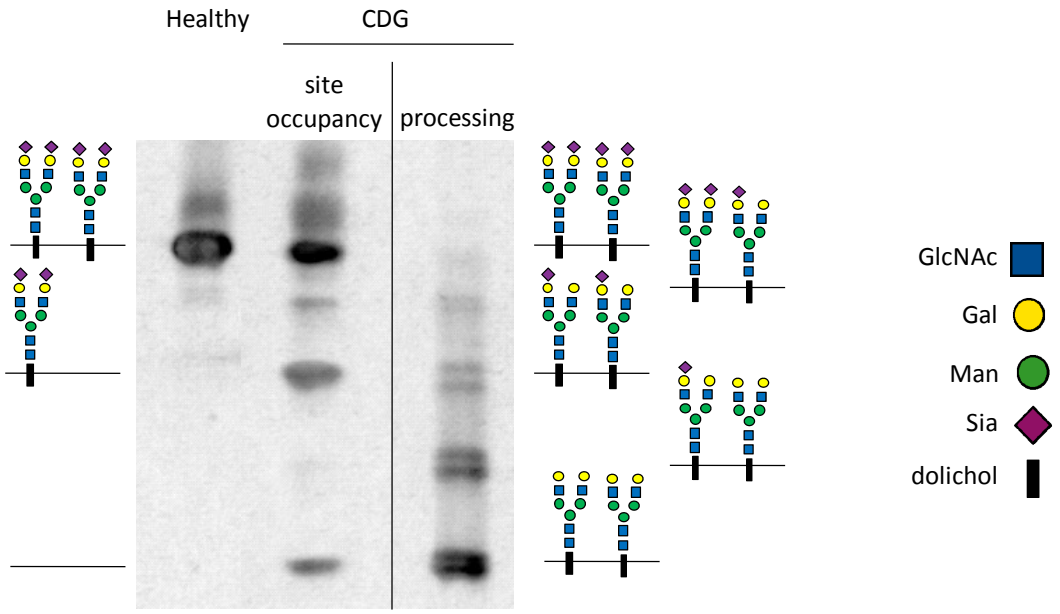

Figure 11 Isoelectric focusing (IEF) of serum transferrin from a healthy individual and from CDG patients.

Transferrin from a healthy individual gives a major band at the position of 4 negative charges. Transferrin from patients with deficiencies in *N*-glycosylation site occupancy present pronounced bands at the position of two and zero charges. Transferrin from CDG patients with defects in *N*-glycan processing shows pronounced bands at any position of underglycosylation. The doubling of bands seen in the last lane is due to an amino acid polymorphism of transferrin and is not due to CDG. Figure adapted from http://www.klinikum.uni-heidelberg.de/6-CDG-Diagnostik.9520.0.html

The symptoms of CDG patients with a defect in *N*-glycosylation are multisystemic. Every cell in the body expresses *N*-glycosylated proteins and thus underglycosylation affects every organ. Brain development is often strongly affected resulting in psychomotor retardation, hypotonia and ataxia. Hormonal and coagulation disorders are also frequent. So are dysmorphic features of the face and hands, inverted nipples and abnormal fat deposits. The symptoms can range from mild to severe (87, 90, 91).

The symptoms of *O*-glycosylation disorders are often more organ specific because some *O*-glycosylation enzymes only modify particular proteins. For example, some muscular dystrophies result from defects in enzymes that mainly glycosylate α-dystroglycan (48, 49). Another example of an *O*-glycosylation defect with organ specific symptoms is multiple cartilaginous exostoses. This disease is caused by a defect in either the *EXT1* or the *EXT2* gene, which build the heterodimer transferase complex synthesising the GAG chain of heparan sulfates. Diminished heparan sulfate levels affect the formation of the cytokine gradients that are important for directional bone growth. Accordingly, benign tumors grow at the end of the long bones in patients with mutations in the EXT genes (92, 93).

In the class of lipid glycosylation disorders, only two gene defects have been identified. A defect in the promoter region of the PIGM gene causes a reduction in expression of PIGM

and therefore decreased GPI synthesis. PIGM codes for an α1,4-Man transferase that adds the first Man to a GPI anchor. The main disease symptoms are venous thrombosis and seizures (94). The other known, affected gene is ST3GAL5 that codes for an α2,3-Sia transferase. It catalyzes the last step of the formation of the ganglioside GM3. Mutations in ST3GAL5 cause Amish infantile epilepsy and patients show mental and psychomotor retardation with frequent seizures (95).

Defects identified in Golgi carbohydrate transporters including the CMP-Sia and GDP-Fuc transporters belong to the class of multiple glycosylation disorders. Multiple glycosylation defects affect the biosynthesis of *N*- and *O*-glycans as well as glycolipids. Another defect affecting all three pathways is disruption of the synthesis of dolichol-P Man. Additionally, defects indirectly affecting glycosylation are found in the group of multiple glycosylation disorders. This group includes mutations that disturb the localisation of Golgi glycosyltransferases. The Conserved Oligomeric Golgi (COG) complex as an example, is probably involved in the retrograde transport of glycosyltransferases in the Golgi apparatus (96). Deficiencies in five of the eight subunits are known to disrupt late Golgi glycosyltransferase localisation and lead to CDG (97, 98). As another example, a mutation in the a2 subunit of the vesicular H^+-ATPase (named ATP6V0A2-CDG) causes an indirect glycosylation defect. It is likely that this mutation disturbs the acidification of the Golgi compartments which in turn affects the distribution and function of glycosyltransferases (99). ATP6V0A2-CDG patients present the particular phenotype of wrinkled skin (cutis laxa) together with the usual *N*-glycosylation disorder phenotypes. Overall, the phenotypes of multiple glycosylation defects are similar to *N*-glycosylation defects with multisystemic features, which mainly affect the central nervous system.

Unfortunately, at present no treatment for CDG exists with the exception of deficiencies affecting phosphomannose isomerase (PMI) and the Fuc-transporter. Feeding of Man to PMI patients and feeding Fuc to patients with a deficiency in the GDP-Fuc transporter improves symptoms and restores normal glycosylation. However, for all other known defects to which most of the patients belong, only palliative care is available (100, 101).

Mis-glycosylation of proteins is not always caused by an inherited mutation in the glycosylation pathway. Altered glycosylation can also be acquired. Several disease states affect glycosylation patterns. The most prominent example is cancer. Cancer cells express altered surface glycosylation (102). These changes are involved in the metastatic process, supporting the extravasation of tumor cells from the blood stream and the invasion of distant organs. Furthermore, changes in cytokine receptor glycosylation alter the activity of the receptor and increase cellular growth (103, 104). These changes are based on altered gene expression of Golgi glycosyltransferases.

Glycosylation is also affected by high doses of alcohol. Chronic alcohol abuse decreases *N*-glycosylation (105). However, neither the molecular mechanisms causing this underglycosylation nor the correlation of underglycosylation to symptoms are understood. Nevertheless, underglycosylation can be used as a marker for alcohol abuse in clinics. Serum transferrin analysis is used for the detection of alcohol abuse as for the detection of CDG (106).

The glycosylation machinery can also be directly inhibited by chemical compounds. Several plants synthesize alkaloids that inhibit glycosidases. Locoweed plants for example, produce swainsonine, which can poison and kill livestock when eaten, or even poison humans via the milk of these animals (107, 108). Swainsonine inhibits Golgi α-mannosidase2 and therefore inhibits the synthesis of complex type glycans. Additionally, it inhibits lysosomal mannosidases and causes an accumulation of glycans in the lysosomes.

Gene mutations do not necessarily have to be inherited as in CDG but can also be acquired. These somatic mutations of the glycosylation machinery have also been observed. Tn syndrome is caused by a mutation in COSMC in hemapoietic stem cells (109). COSMC is a chaperone required for the correct folding of, and therefore the activity of core 1 β1,3-Gal transferase (110). The resulting truncated *O*-glycan structures on blood glycoproteins lead to an autoimmune response and to the lysis of blood cells.

Perspectives in *N*-glycosylation and CDG research

To date, 15 genes are known to cause solely *N*-glycosylation disorders and 15 genes are known to cause disorders affecting *N*- and other glycosylation pathways (table 1 and (88)). There are still several genes of the *N*-glycosylation machinery that are not yet connected to a disease and are potential candidates for new CDG. Dolichol kinase is the only gene in the dolichol synthesis and recycling pathway so far reported to have a disease causing mutation (111). It is possible that mutations in the biosynthetic enzymes preceding dolichol phosphorylation such as the dolichol reductase will eventually be found to cause CDG. CHO cells with a mutation in the dolichol reductase gene show a strong reduction in LLO synthesis (112). There are also CDG candidate genes in the dolichol recycling pathway. Disruption of the yeast homolog for Dolichol-P-P-phosphatase causes an underglycosylation of proteins making this gene a strong candidate (113, 114).

It is likely that deficiencies in the Glc-P-dolichol transferase (*ALG5*), the α1,2-Man transferase (*ALG11*), the α1,2-Glc transferase (*ALG10*), and the second β1,4-GlcNAc transferase (*ALG13/ALG14*) cause CDG. It is also expected that mutations in OST genes affecting the efficiency of transfer of the glycan to the protein cause underglycosylated proteins. So far only deficiencies in the subunits TUSC3 and MAGT1 have been reported (115, 116). These patients presented non-syndromic mental retardation. Surprisingly, these two defects only affect particular *N*-glycoproteins. The nonspecific clinical phenotype and the fact that underglycosylation cannot be detected on transferrin in these deficiencies suggests that defects in the OST may not always be recognized.

In the processing and elongation steps of the *N*-glycan, glucosidase1 and β1,2-GlcNAc transferase2 are known to cause CDG. Mutations have been reported in the glucosidase2 gene, and the corresponding disease is called autosomal dominant polycystic liver disease (117). However, this disease does not present underglycosylation and therefore is not a member of the CDG. There are no mutations reported for the β1,2-GlcNAc transferase1 gene, which initiates the production of hybrid and complex type *N*-glycans. Functioning prior to β1,2-GlcNAc transferase2, a deficiency in the β1,2-GlcNAc transferase1 gene would be likely to cause CDG.

Deficiencies affecting *N*-glycan elongation have been reported for other genes. However, these defects also affect other classes of glycosylation, placing them in the class of multiple

glycosylation disorders. It is likely that the remaining subunits of the COG complex will be implicated in unresolved cases of CDG. In addition, proteins involved in trafficking, such as SNARE proteins, GTPases, or coat proteins can be imagined to affect trafficking of glycosyltransferases to the Golgi apparatus (118). Indeed, very recently, the cause of congenital dyserythropoietic anemia type II in some patients was attributed to mutations in the SEC23B gene, a coat component of COPII vesicles (119).

Genes for proteins that regulate the pH gradient in the Golgi apparatus such as the H^+-ATPase, are another class of genes likely to be associated with untyped CDG. One potential pH regulating candidate gene is GPHR, an anion-channel, probably transporting chloride ions (120). Chloride ions are necessary as counter-ions for protons that are pumped into the Golgi apparatus. It has been shown that GPHR deficient cell lines present underglycosylation of different marker proteins (120).

Phosphate and manganese concentration can also affect the Golgi environment. Inorganic phosphate is the byproduct of many glycosylation reactions and has to be transported out of the Golgi apparatus (25). Manganese is an essential cofactor for most glycosyltransferases. Defects in manganese or phosphate transport into and out of organelles could inhibit glycosylation reactions (121, 122).

In addition to the discovery of new genes causing CDG, it is important to find therapies for patients. In order to find potential therapeutic targets, we must understand the correlation between the missing glycan and the resulting phenotype. Knowledge about altered signaling pathways and inactivated proteins caused by misglycosylation can reveal possible therapeutic targets.

Knockout mice are important tools to investigate the consequences of a deletion of particular genes and to investigate new drugs and therapies. However, for the LLO synthesis pathway and the early processing enzymes of *N*-glycosylation, it is impossible to produce knockout mice because deletions are embryonic lethal. Knock-in of known mutations in mice is one possibility to generate models for CDG. At the cellular level, primary fibroblast cultures and cell culture models of CDG are helpful tools to investigate CDG and to test potential drugs.

Concluding remarks

Carbohydrates modifying proteins and lipids play a crucial role in many physiological processes from development, to immunology, to protein folding, targeting and stability. Investigation of the structure, synthesis and function of glycans adds to our understanding of many biological processes. Diseases of glycosylation highlight the importance of glycosylation for life. Studying glycosylation disorders makes it possible to investigate where, when and how glycans play a role in the human body.

Aims of the study

For CDG patients it is important to know the aetiology of the disease and the defective gene. Even if no medical treatment is available, it is important to exclude other diseases by identifying the underlying CDG causing mutation. Additionally, prenatal diagnostics will be an increasingly common instrument for parents to decide whether to continue a pregnancy in the case of severe untreatable disease. In the first part of this work, the genetic defect of an untyped CDG case was investigated. The patient presented typical, however mild symptoms of CDG. The IEF pattern showed a weak underglycosylation with bands at three and two Sia, pointing to a defect in *N*-glycan processing. Furthermore, *O*-glycosylation was also affected. These observations suggested a COG defect. Therefore, we investigated retrograde transport in patient fibroblasts, identified the disease causing mutation, and complemented the patient fibroblasts with the wildtype gene.

While it is important to identify the cause of a disease, it is also necessary to understand the correlation between a mutation and the phenotype. The second and the third part of this thesis focus on investigations of the cellular responses to underglycosylation.

In the second part, different phenotypes of CDG patients with mutations in the two ER glucosyltransferases ALG6 and ALG8 were investigated. Despite the fact that the ALG6 glucosyltransferase functions prior to the ALG8 glucosyltransferase in the LLO synthesis pathway, the phenotype of the ALG6-CDG patients is milder than the phenotype of the ALG8-CDG patients. Furthermore, no mutation was known for the last ER glucosyltransferase ALG10. To investigate if ALG8 has a function other than transferring the second glucose to the LLO, we silenced each of the three genes by RNAi in HeLa cells. We compared the

unfolded protein response and the secretion and glycosylation of proteins in the three knockdowns with wildtype HeLa cells.

In part three, I describe our investigation of altered gene expression in response to underglycosylation in different CDG fibroblasts. Underglycosylation of receptors is suggested to alter signaling pathways, and this altered signaling may be the underlying cause of some of the symptoms. To identify altered gene expression in CDG patients, the global mRNA expression profile of three different CDG was compared to healthy controls by genearray. Extracellular matrix (ECM) genes were highly induced in CDG patients. This observation was confirmed on the protein level. The involvement of the TGFβ pathway and IGFBP5 in the induction of ECM genes was investigated.

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Results

Manuscripts:

Deficiency in COG5 causes a moderate form of congenital disorders of glycosylation

(Hum. Mol. Genet.: 2009 Nov 15; 18(22):4350-6)

The impact of ER glucosyltransferase deficiency onto glycoprotein secretion in HeLa cells

(in preparation)

Fibrotic response in fibroblasts from congenital disorders of glycosylation

(in preparation)

Deficiency in COG5 causes a moderate form of congenital disorders of glycosylation

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Abstract

The conserved oligomeric Golgi (COG) complex is a tethering factor composed of eight subunits that is involved in the retrograde transport of intra-Golgi components. Deficient biosynthesis of COG subunits leads to alterations of protein trafficking along the secretory pathway and thereby to severe diseases in humans. Since the COG complex affects the localization of several Golgi glycosyltransferase enzymes, COG deficiency also leads to defective protein glycosylation, thereby explaining the classification of COG deficiencies as forms of congenital disorder of glycosylation (CDG). To date, mutations in *COG1*, *COG4*, *COG7* and *COG8* genes have been associated with diseases, which range from severe multi-organ disorders to moderate forms of neurological impairment. In the present study, we describe a new type of COG deficiency related to a splicing mutation in the *COG5* gene. Sequence analysis in the patient identified a homozygous intronic substitution (c.1669-15T>C) leading to exon skipping and severely reduced expression of the COG5 protein. This defect was associated with a mild psychomotor retardation with delayed motor and language development. Analysis of different serum glycoproteins revealed a CDG phenotype with typical undersialylation of N- and O-glycans. Retrograde Golgi-to-endoplasmic reticulum trafficking was markedly delayed in the patient's fibroblast upon brefeldin-A treatment, which is a hallmark of COG deficiency. This trafficking delay could be restored to normal values by expressing a wildtype COG5 cDNA in the patient cells. This case demonstrates that COG deficiency and thereby CDG must be taken into consideration even in children presenting mild neurological impairments.

Introduction

Glycosylation is an ubiquitous form of post-translational modification that is essential in most living organisms. In humans, defects of glycosylation cause diseases classified under the generic name of congenital disorders of glycosylation (CDG). The features of these diseases reflect the broad physiological relevance of glycans, thereby ranging from multi-organ failures, dysmorphisms and skeletal malformations to hormonal disorders and coagulopathies (1-3). The clinical manifestations depend also on the type of glycosylation affected. Defects of N-glycosylation are rather associated with neurological disorders, whereas defects of O-mannosylation are linked to congenital muscular dystrophies (4) and defects of mucin-type O-glycosylation for example with anemia (5) and tumoral calcinosis (6).

Diagnosis of CDG is usually reached by isoelectric focusing (IEF) of the serum proteins transferrin and apolipoprotein CIII (ApoCIII) (7) followed by specific enzymatic assays and mutation analysis (8, 9). Most forms of CDG can be assigned to mutations in genes participating in glycan biosynthesis including glycosyltransferases, glycosidases, sugar transporters and enzymes involved in the production of substrates for the glycosylation reactions. Only recently, defects in genes involved in vesicular trafficking have been shown to cause CDG. Mutations in the genes encoding subunits of the conserved oligomeric Golgi (COG) complex were the first group of CDG defects to be found outside of the glycosylation pathway (10-12). The COG complex has been described as a cytosolic protein complex that is peripherally associated with the Golgi serving as a tethering factor for retrograde vesicular transport.

To date, mutations in genes encoding four of the COG subunits, namely COG1, COG4, COG7 and COG8, have been described as causing CDG (13-17). In COG-deficient cells, the intra-Golgi retrograde transport is disturbed, which affects the distribution of so-called GEAR proteins (18). The GEAR protein family includes SNARE proteins, the golgin and giantin matrix proteins and glycosyltransferases and glycosidases, such as α-mannosidase II, β1,2-*N*-acetylglucosaminyltransferase I, α-2,3-sialyltransferase I (13, 18, 19). Because of the abnormal distribution of several glycosylation enzymes, COG deficiency is usually associated with underglycosylation of proteins and thus with typical CDG phenotypes. Besides leading to glycosylation defects, COG deficient cells have previously been shown to respond slowly

to brefeldin-A (BFA) treatment (20). This drug blocks the GDP/GTP-exchange factor of ADP-ribosylation factor 1 and inhibits the formation of COPI vesicles on the Golgi membrane. It leads to a rapid redistribution of Golgi proteins into the endoplasmic reticulum (ER) (21), a process that can be visualized by immunofluorescence microscopy of Golgi resident proteins. Considering the combined impairment of vesicular trafficking and protein glycosylation, it is expected that COG deficiency leads to severe diseases. However, as reported here, it appears that COG deficiency can also be associated with relatively mild clinical features.

Results

The index patient is the first child of remotely consanguineous parents of Iraqi origin. At her first neuropsychological examination at the age of 8 years, she showed global developmental delay with moderate mental retardation (IQ around 50-55). Her speech was slow and inarticulate. She had a tendency to tumble and displayed truncal ataxia and slight muscular hypotonia. Magnetic resonance imaging analysis showed pronounced diffuse atrophy of the cerebellum and brain stem (data not shown). The supratentorial brain parenchyma was without pathological findings. Karyotype analysis, routine laboratory tests and screens for inborn errors of metabolism including thyreotropin-releasing-hormone, α-fetoprotein, very-long-chain fatty acids and vitamin E were normal. At the age of 12, IEF of serum transferrin was performed, leading to the first indication for CDG (Fig. 1A). Now at 14 years, she has a good speech perception, even tough her speed of speech is still slow. Her language skills improved significantly and her education is bilingual (Arabic/German). She remains mildly hypotonic, with normal reflexes, good strength and truncal ataxia without extrapyramidal signs. Blood for genomic DNA analysis was drawn from all family members, whereas a skin biopsy was taken only from the index patient.

The IEF of serum transferrin in the patient showed increased levels of trisialo-transferrin that clearly differed from the pattern of a control subject or a patient with a N-glycosylation defect caused by a phosphomannomutase2 (PMM2) deficiency (Fig. 1A). This accumulation of trisialo-transferrin is usually a sign of normal N-glycosylation site occupancy but incomplete N-glycan structures. To investigate the N-glycan structure, we have analyzed serum transferrin using electrospray ionization mass spectrometry (Fig. 1B). The observed transferrin peaks were assigned according to the calculated molecular masses (22). In the patient sample, an elevated peak at 79'248 Da was observed. The mass shift of about 300 Da indicated the loss of a single sialic acid residue in the glycoprotein. This finding was compatible with the result obtained from the IEF analysis.

Considering the modest underglycosylation of serum transferrin, we assessed additional serum glycoproteins such as haptoglobin and α1-acid glycoprotein (AGP). Haptoglobin normally carries four N-glycans (23), whereas haptoglobin from a PMM2-deficient patient shows underglycosylated protein forms missing complete N-glycans (Fig. 1C). However, haptoglobin from the present patient did not show any loss of N-glycans, although it

appeared to migrate slightly faster in the SDS-PAGE compared to the control lane, suggesting truncated glycan structures on the protein of the patient (Fig. 1C). Enzymatic release of N-glycans with the endoglycosidase PNGaseF led to identically deglycosylated haptoglobin in all samples (Fig. 1C). The glycosylation status of AGP was investigated by 2-D gel electrophoresis. AGP contains five highly sialylated complex-type N- glycans (24), and, due to its low pI narrow-range IEF it can be analyzed readily without purification steps (25). The molecular masses of the AGP glycoforms were comparable in both control and patient samples. However, a shift to more basic pI values was observed in the patient sample (Fig. 1D). This shift was compatible with reduced terminal sialylation of the patient AGP. To determine whether reduced sialylation was limited to N-glycans, we also analyzed the sialylation of the serum O-glycoprotein ApoCIII. The IEF of control and patient serum ApoCIII revealed the presence of unsialylated ApoCIII in the patient sample (Fig. 1E).

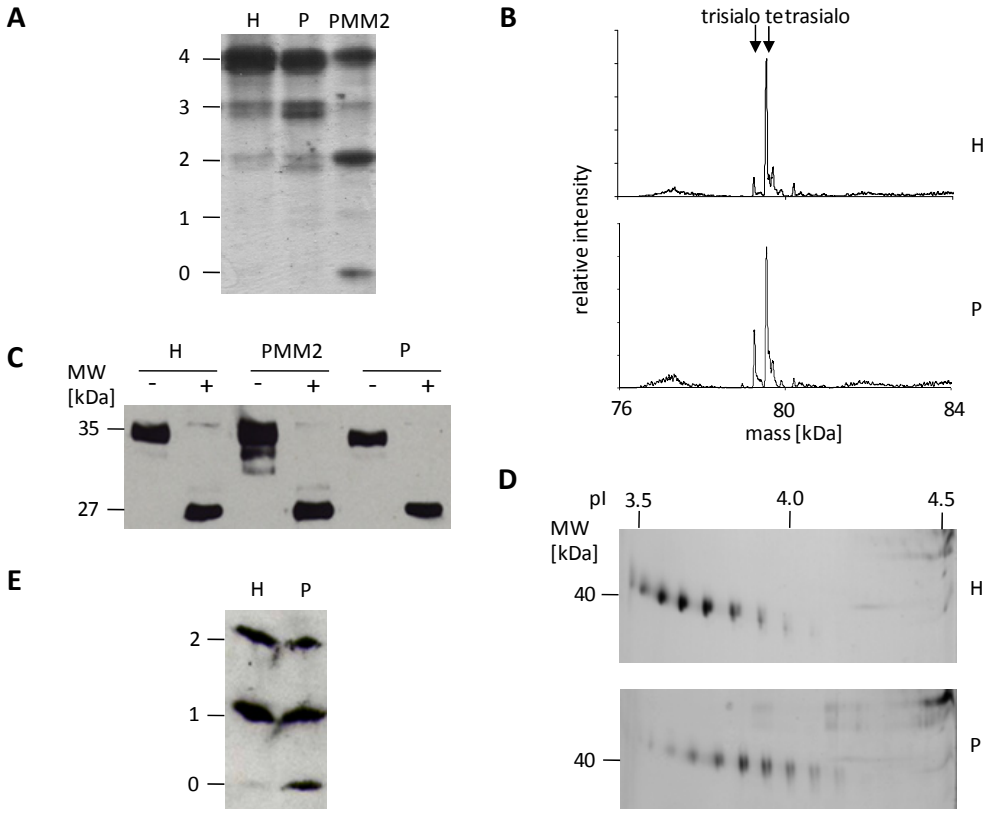

Figure 1 Glycosylation of serum glycoproteins.

A. IEF of transferrin from healthy control (H), patient (P) and PMM2 deficient patient (PMM2). The number of sialic acid residues is given on the left. **B.** Electrospray ionization mass spectrometry analysis of transferrin from healthy control (H) and patient (P). The deconvoluted mass spectra with the molecular masses (kDa) and the relative intensity are indicated. The fully glycosylated transferrin (tetrasialo) and the lighter trisialo-transferrin are marked with arrows. **C.** Western blot analysis of haptoglobin in healthy control (H), PMM2 deficient patient (PMM2) and patient (P), before (-) and after (+) PNGaseF treatment. **D.** Narrow range 2-D gel electrophoresis of AGP from healthy control (H) and patient (P). Molecular weight (MW) and isoelectric point (pI) are indicated. **E.** IEF and immunoblotting of ApoCIII of healthy control (H) and patient (P). The number of sialic acid residues is marked at the left.

A defect of terminal sialylation of both N- and O-glyans has been previously described in patients with deficiency of the COG complex (11, 26). COG deficiency is associated with delayed BFA-mediated collapse of the Golgi apparatus into the ER (20), which represents a simple test to assess a possible COG involvement in the underglycosylation of the present patient. Treatment of control and patient fibroblasts with BFA and monitoring of the Golgi structure by β1,4 galactosyltransferase-I immunofluorescence confirmed the possibility of a COG deficiency, as shown by the BFA-resistant phenotype in the patient cells (Fig. 2).

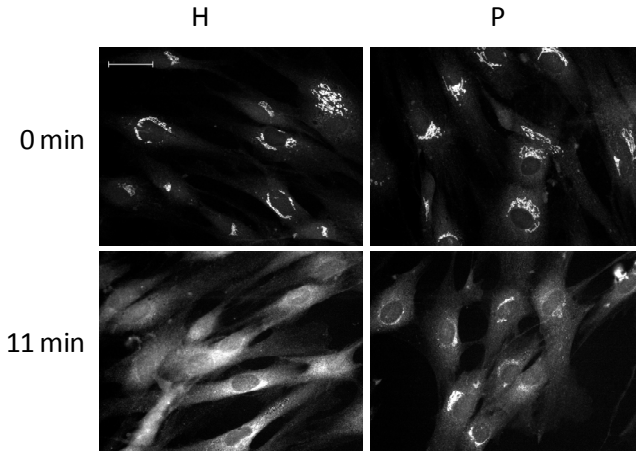

Figure 2 Retrograde transport in BFA treated cells.

Healthy control (H) and patient (P) fibroblast were treated with BFA for the indicated time. The Golgi apparatus was stained with β1,4 galactosyltransferase-I antibody. The scale bar corresponds to 20 μm.

To detect possible mutations in COG genes, we first amplified the corresponding transcripts by RT-PCR. In this survey, we noticed an unexpectedly short cDNA besides the normal *COG5* cDNA. Using various primer sets allowing the amplification of partial stretches of the *COG5* mRNA, we localized the cause of the shorter transcript to a region of the *COG5* gene encompassing exons 13 to 18 (Fig. 3A and B). Direct sequencing of both *COG5* cDNAs revealed the skipping of exons 15 and 16 in the shorter transcript. Sequence analysis of the patient genomic DNA identified a homozygous mutation in the intronic region 15 bp upstream of exon 15 (c.1669-15T>C), which is presumably leading to the observed altered splicing. To exclude common single nucleotide polymorphisms, we screened 200 unrelated alleles using a MALDI-TOF-MS-based procedure for polymorphism genotyping (27). Fifty of these alleles were derived from samples of similar ethnical and geographical region to the patient's family. None of the alleles analyzed carried the COG5 mutation c.1669-15T>C (data not shown). Haplotype analysis of the family validated our findings since the parents both

carry the intronic mutation (Fig. 3C). The expression of the shorter transcript was calculated to create a loss of 58 amino acids (6.4 kDa) in the COG5 protein since the loss of exons 15 and 16 does not lead to a frameshift during translation. To examine this finding, we analyzed the size and amount of the COG5 protein by Western blotting in control and patient fibroblasts. As shown in Fig. 3D, the low level of full length COG5 protein detected in the patient fibroblasts confirmed the disease-causing effect of the splicing mutation. However, no shorter protein was observed, suggesting that the truncated protein was unstable and prone to degradation.

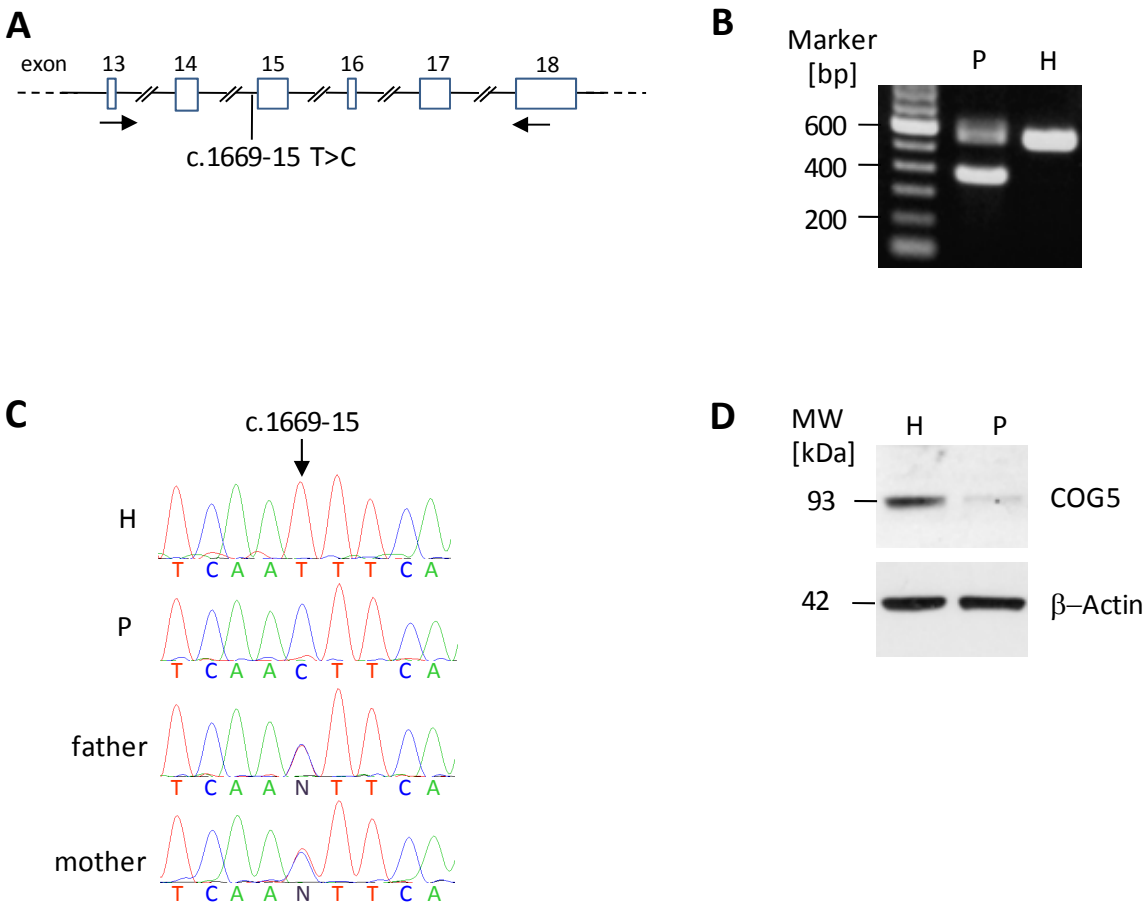

Figure 3 ***COG5*** **mutation analysis.**

A. Schematic representation of the *COG5* gene organization surrounding the mutation. The positions of the primers used in the RT-PCR and of the mutation are indicated. **B.** RT-PCR analysis of mRNA isolated from healthy control (H) and patient (P) fibroblasts. The primers used span exons 13 to 18. The healthy control cDNA fragment is 557 bp-long. The RT-PCR product from the patient sample showed an additional, shorter fragment of 383 bp. The bp-marker is shown at the left. **C.** Electropherograms corresponding to the stretch of *COG5* genomic DNA encompassing the c.1669-15T>C mutation as detected from healthy control DNA (H), patient DNA (P) and DNA from the parents of the patient. The mutation is indicated by an arrow. **D.** Steady-state levels of the COG5 protein detected by Western blotting from healthy control (H) and patient (P) fibroblasts. β-actin was used as loading control.

To address whether the detected *COG5* mutation was uniquely responsible for the trafficking defect, we introduced a wildtype *COG5* cDNA by lentivirus-mediated transfection in the patient fibroblasts. After confirming the stable expression of the wildtype *COG5* construct in the fibroblasts (data not shown), we treated the cells with BFA and monitored the collapse of the Golgi apparatus as done before. Whereas the patient cells transfected with a mock lentivirus construct showed the previously described delayed collapse of the Golgi apparatus, the patient cells expressing the normal *COG*5 cDNA showed the same rapid collapse of the Golgi apparatus as normal cells, thereby confirming the causality of the COG5 deficiency in the observed phenotype (Fig. 4). As a control, we also showed that expression of either a mock or a *COG5* construct in normal fibroblasts did not significantly affect the speed of the collapse.

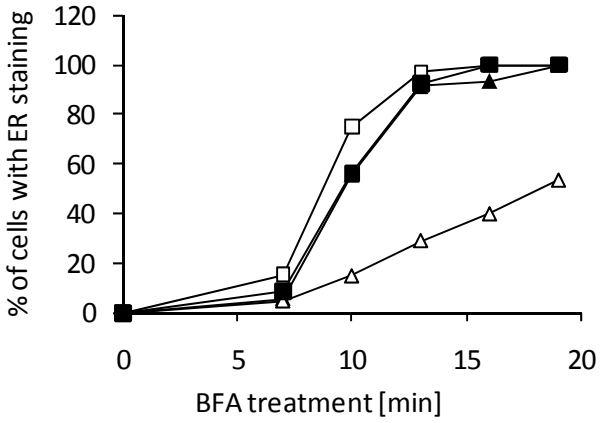

Figure 4 Retrograde transport in fibroblast cells expressing a wildtype COG5 cDNA.

Healthy control (squares) and patient (triangles) fibroblasts were lentivirally transduced with either a mock (opened) or a *COG5* cDNA (closed) construct. The Golgi apparatus was stained with β1,4 galactosyltransferase-I antibody. For each time point 250 cells were counted and the values expressed in percents of cells with an ER-staining pattern.

Discussion

The identification of forms of CDG caused by COG deficiency marked a new era in CDG pathogenesis, since these defects affect glycosylation indirectly through altered trafficking of glycosyltransferases. To date, deficiencies in the COG1, COG 4, COG 7 and COG 8 subunits have been related to a form of CDG. The first case identified, namely COG7 deficiency, was described for two siblings who died in their first months of life (13). Subsequently identified COG7 patients were also severely affected so that it has been concluded as COG7 deficiency is mainly a lethal multisystemic disorder (28, 29). Two patients are known to be affected at the *COG8* locus (15, 16). The clinical features were not as severe as those observed in COG7 deficiency, but both COG8 patients were strongly retarded. They showed obvious neurological abnormalities and required hospitalization. Up to now, the COG1 patients and the recently published COG4 case have represented the group with the mildest clinical features (14, 17, 21, 26, 30). However, the present case of COG5 deficiency was even milder, since her clinical status allowed her to live a regular life and to attend a college with therapeutic support.

The moderate clinical outcome of the present patient might be explained by the rather mild glycosylation defect observed in the COG5-deficient cells, since only terminal sialylation was affected. This glycosylation phenotype was also seen in COG5-depleted HeLa cells (31). The mild outcome of COG5 deficiency was also noted in loss of COG5 function in *D. melanogaster*, which affected spermatogenesis, while no other apparent phenotypes were reported (32). In contrast, analysis of patient cells with COG1 and COG7 defects revealed that β1,4-linked galactose residues were also partially missing in addition to lacking terminal sialylation (17). COG subunits may each mediate interaction with different set of proteins, thus explaining varying severity of defects. Defect at a single subunit does not obligatorily destabilize the whole complex. Along this line, it has been shown that deficiency of the TRAPPC2 subunit of the TRAPP complex, another tethering factor, leads to a spondyloepiphyseal dysplasia tarda, a disease affecting the secretion of some extracellular matrix proteins (33).

The intronic mutation in the *COG5* allele is leading to partial skipping of exons 15 and 16 and sequence analysis of the shorter transcript suggested the expression of a truncated COG5 protein in the patient cells, especially because the shorter transcript was more abundant.

However, only the full length protein was detected upon Western blot analysis. We assume that the shorter COG5 protein is unstable and thus leading to its degradation.

The description of additional cases of COG deficiency will eventually establish whether the severity of COG deficiency relates to the affected COG subunit or rather to the level of inactivation conferred by individual mutations irrespectively of the subunits themselves.

Since the splicing mutation described here enabled the expression of full-size COG5 protein, yet to low levels, we would assume that other mutations may lead to more severe forms of COG5 deficiency. The identification of a novel mutation in the COG complex is important, since only eleven patients with defects in four of the subunits of the complex have been described so far. This emphasises that probably all the subunits are essential for correct Golgi trafficking and glycosylation and it is likely that mutations in all subunits of the COG complex could be identified soon among untyped CDG cases. The present description of the COG5 defect in this patient calls for a revision of the concept that COG deficiencies represent rather severe forms of CDG. Accordingly, we would recommend testing for possible COG defects also among cases of mild neurological disorders.

Materials and methods

Glycosylation analysis of serum glycoproteins - IEF of transferrin was performed as described (7) using Immobiline DryPlates (pH 4–7, GE Healthcare) and the Phast System (GE Healthcare). Two-dimensional SDS-PAGE for AGP analysis was carried out as described by Kleinert et al. (25). Transferrin was isolated from serum samples and analyzed with electrospray ionization mass spectrometry as reported (22).

Antibodies and Western blotting - Anti-haptoglobin was purchased from Rockland (Gilbertsville, PA, USA) and anti-ApoCIII from ANAWA (Wangen, CH). Anti-COG5 was a gift from Dr. D. Ungar (University of York, York, UK) and was used at a dilution of 1:100. Proteins were separated by SDS-PAGE and immunoblotted. Signals were detected using the ECL detection kit (Amersham Biosciences). Enzymatic deglycosylation of serum haptoglobin was performed using PNGase F (New England Biolabs).

Sequencing and mutation analysis of COG5 gene - Total RNA was isolated from fibroblasts using Qiagen RNeasy Kit (Qiagen Inc.). Reverse transcription was performed using QIAGEN OneStep RT-PCR Kit. Mutation screening of the *COG* genes *COG1* to *COG8* was carried out by fluorescent sequencing of cDNA-amplified PCR products on an ABI 3100 automated sequence detection system (Applied Biosystems). PCR conditions and primer sequences are available from the authors upon request. Genomic DNA was isolated from peripheral blood leukocytes using DNeasy Blood & Tissue Kit (Qiagen Inc.). For amplification of the *COG5* cDNA region that was used to examine the skipping of exon 15 and 16, respectively, the following primers were used: 5'- GATTATGATCCAGAAAAGGCT -3' (forward) and 5'-GGATAATGACCCAGAAAAGTC -3' (reverse). These primers span the exons 13 – 18. For amplification of the genomic region that includes the mutation identified in this study, the following primers were used: 5’- TAACATTCTCTTTGTCAGA -3’ (forward) and 5’-GCCAGAGTCAAAGACTGT -3’(reverse). The exact PCR protocols are available on request.

Single nucleotide polymorphism analysis - The genomic DNA region containing the mutation was amplified using the forward and reverse primer pair 5'-TAACATTCTCTTTGTCAGA-3' (forward) and 5'-GCCAGAGTCAAAGACTGT-3' (reverse). The PCR product was purified using

the QIAquick PCR purification kit (Qiagen Inc.) and used as template for the allele-specific primer extension. The extension reaction was performed with the extension primer 5'-TCATTCTGACACATTCTLTTTGAA-3' containing a photocleavable linker. The exact PCR protocols are available on request. The extended product was purified using the genopure oligo kit (Bruker Daltonics) according to the manufacturer's instructions. The sample was transferred to an AnchorChip sample target (400 μm, Bruker Daltonics) preloaded with 3-HPA matrix. After UV-cleavage of the linker, molecular masses of the extension products were determined with an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics) in the linear mode.

Cell culture - A punch biopsy of skin from the upper arm was taken from the index patient, minced and incubated in DMEM medium with 10% fetal calf serum, 0.5% penicillin and streptomycin (Invitrogen) at 37°C under 5% CO_2. Primary fibroblasts cultures were obtained by expansion of fibroblast cells growing out of skin explants after 7-14 days. The fibroblasts were further cultured in DMEM with 10% fetal calf serum and 0.5% penicillin and streptomycin at 37°C.

BFA assay and immunofluorescence microscopy - Fibroblasts were grown over night on glass cover slips to about 60 % confluence. Medium was then changed with pre-warmed medium containing 2 μg/ml of BFA (LC laboratories). The assay was stopped at different time points by fixing the cells with 3% paraformaldehyde. The cells were permeabilized with 0.1% saponin in PBS followed by immunostaining using a mouse monoclonal antibody to the human β1,4 galactosyltransferase-I protein in a 1:50 dilution (gift from Dr. E. G. Berger, University of Zurich, Switzerland). Anti-mouse Alexa Fluor-488 antibody was purchased from Molecular Probes (Invitrogen) and pictures were taken with a fluorescence microscope (Axiovert 200, Zeiss).

Cloning of COG5 and transfection of fibroblasts - The vector encoding *COG5* cDNA was obtained from Open Biosystems (MHS1010-97227871). The terminal 113 bp of *COG5* missing in this construct were introduced as a *BamH*I-*Xba*I PCR fragment amplified from fibroblast cDNA with the following primer: 5'-TTGGATCCTCCAGCTCAGGAAAACCTGATG-3' (forward,

including a *BamH*I restriction site) and 5'-CGTCTAGAGGGTTAGCACAAAGTGGAGATG-3' (reverse, including a *Xba*I restriction site). The complete *COG5* cDNA was then subcloned into pLenti6 vector (Invitrogen) using *BamH*I and *Sac*II restriction sites. Transfection of fibroblasts using lentiviral vectors was carried out as described previously (34).

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Conflict of interest statement

No conflict of interest declared.

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The impact of ER glucosyltransferase deficiency onto glycoprotein secretion in HeLa cells

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Abstract

Congenital disorders of glycosylation (CDG) are caused by mutations in the glycosylation machinery. CDG patients mainly present with neurological symptoms of variable severity. The majority of CDG defects described to date affect the biosynthesis of lipid-linked oligosaccharides leading to deficient N-glycosylation. The biosynthesis of lipid-linked oligosaccharides in the endoplasmic reticulum finishes with the addition of three glucose residues by the ALG6, ALG8 and ALG10 glucosyltransferases. These glucose residues are required for the recognition of lipid-linked oligosaccharide by the oligosaccharide transferase complex and, after transfer to proteins, for proper folding of glycoproteins. To investigate the impact of defective glucosylation on the glycosylation and secretion of glycoproteins, we have disrupted ALG6, ALG8 and ALG10 gene expression by shRNA mediated inactivation in HeLa cells. The decreased glucosyltransferase expression led to the expected accumulation of incomplete lipid-linked oligosaccharides. However, both the N-glycosylation and processing of the test proteins α1-antitrypsin and cathepsin D were not altered in the three glucosyltransferase-deficient cell lines. Furthermore, markers of the unfolded protein response were not induced in the glucosyltransferase-deficient cell lines. The investigation of the unfolded protein response markers in primary CDG fibroblasts deficient for ALG6 and ALG8 showed the same pattern of unchanged expression. This study shows that N-glycosylation and processing of glycoproteins in HeLa cells are not significantly affected by ER glucosyltransferase deficiency.

Introduction

N-glycosylation is a widespread and complex form of protein modification in eukaryotes. N-glycans fulfill important roles both intracellularly and extracellularly. In the endoplasmic reticulum (ER), N-glycans act as a signal in the process of glycoprotein folding and translocation (1). At the cell surface or on secreted glycoproteins, N-glycans act as ligands involved in cell adhesion, migration and activation (2-4). The N-glycosylation pathway begins in the ER by the assembly of lipid-linked oligosaccharides (LLO) on the carrier dolichol-pyrophosphate (DolPP). After completion of the DolPP-$GlcNAc_2Man_9Glc_3$ structure, the oligosaccharide moiety is transferred to the nascent polypeptide chain by the oligosaccharide transferase (OST) complex (5). The trimming of the three terminal glucose residues in the ER plays a key role in the quality control of glycoprotein folding (1). The early assembly and cleavage of N-glycans is highly conserved in eukaryotes from yeast to human (6).

Mutations in genes of the LLO biosynthesis pathway lead to the accumulation of incomplete LLOs and to decreased occupancy of N-glycosylation sites in newly synthesized proteins. In humans, such defects of LLO biosynthesis lead to diseases known as congenital disorders of glycosylation (CDG) (7, 8). The symptoms associated with CDG are multi-systemic often affecting the central nervous system (7, 9). The impairment of the patients ranges from mild to severe, sometimes with lethal outcome during infancy. In humans, the severity of a given form of CDG does not correlate with the position of the gene defect along the LLO biosynthesis pathway as it is seen in yeast. Accordingly mutations in proximal and distal genes of the pathway yield diseases that can often not be differentiated by clinical examination.

The ER glucosyltransferases ALG6, ALG8 and ALG10 catalyze the last three steps of the LLO synthesis before the LLO is transferred by the OST complex to a N-glycosylation site of a newly synthesized protein (10). In humans, mutations in the ALG6 and ALG8 genes are known to cause CDG (11, 12). Surprisingly, the symptoms of ALG8 deficiency are far more severe than those of ALG6 deficiency (12-17). By contrast, no case of ALG10 deficiency has been identified yet. The unexpected severity of ALG8 deficiency suggests that the ALG8 protein or its product DolPP-$GlcNAc_2Man_9Glc_2$ may play additional roles in cellular processes besides the sole biosynthesis of the growing LLO. In support of this idea, it has been shown

that the LLO DolPP-$GlcNAc_2Man_9Glc_2$ is a substrate of the trimming ER glucosidase II, which normally acts on protein bound N-glycans (14) (Fig. 1). The glucosidase II enzyme requires the binding of two distinct glycans in order to remove a terminal glucose on a protein bound N-glycan (18-21). Thereby, it is possible that LLOs participate in this reaction as co-factors when monoglycosylated proteins are processed by glucosidase II. A deficiency of the glucosidase II activity would probably affect the quality control of glycoprotein folding and possibly result in the accumulation of misfolded proteins.

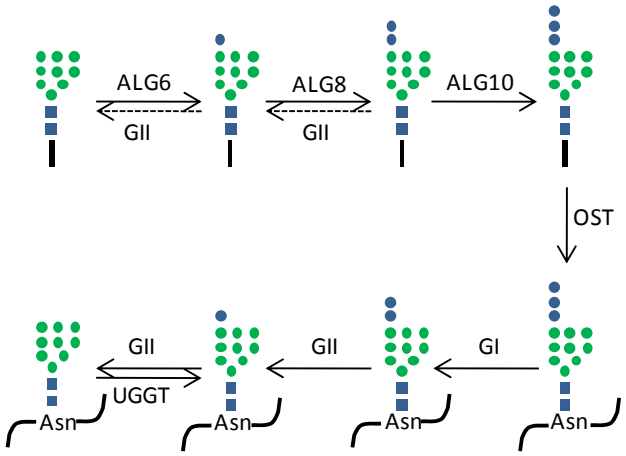

Figure 1 Schematic overview of the ER glucose transfer and cleavage.

The three glucosyltransferases ALG6, ALG8 and ALG10 transfer sequentially three glucose molecules from DolP-glucose to the precursor LLO DolPP-$GlcNAc_2Man_9$. The OST transfers, with high priority, the mature form of the LLO to the protein. Just after the transfer, membrane bound glucosidase I (GI) cleaves the first glucose, thereafter soluble glucosidase II (GII) can cleave the second and third glucose residues. The monoglucosylated protein is substrate for the quality control machinery. The folding sensor UDP-Glc:glycoprotein glucosyltransferase (UGGT) re-glucosylates the protein when not correctly folded. Correctly folded proteins are transferred to the Golgi. Black rectangle: DolPP, blue square: *N*-acetylglucosamine, green circle: mannose, blue circle: glucose

To investigate the impact of ER glucosyltransferase deficiency on N-glycosylation and glycoprotein secretion, we have established knockdown models of the ALG6, ALG8 and ALG10 genes in HeLa cells. The phenotypes of these knockdown cell lines were compared to those observed in primary fibroblasts isolated from ALG6 and ALG8 deficient patients.

Results

To obtain a stable cell model system for deficiency of ER glucosyltransferases, HeLa cells were transfected with lentivirus expressing shRNA fragments that decreased the mRNA levels of the ALG6, ALG8 and ALG10 genes. Whereas no ALG8 mRNA could be detected in shRNA expressing HeLa cells as detected by Northern blotting, the reduction of ALG6 and ALG10 expression was not complete (Fig. 2A). The analysis of mRNA levels by quantitative RT-PCR confirmed the residual mRNA levels detected in the knockdown HeLa cells. In these cells, the mRNA levels reached for ALG6 33%, ALG8 3% and ALG10 16% of the respective normal levels (Fig. 2B).

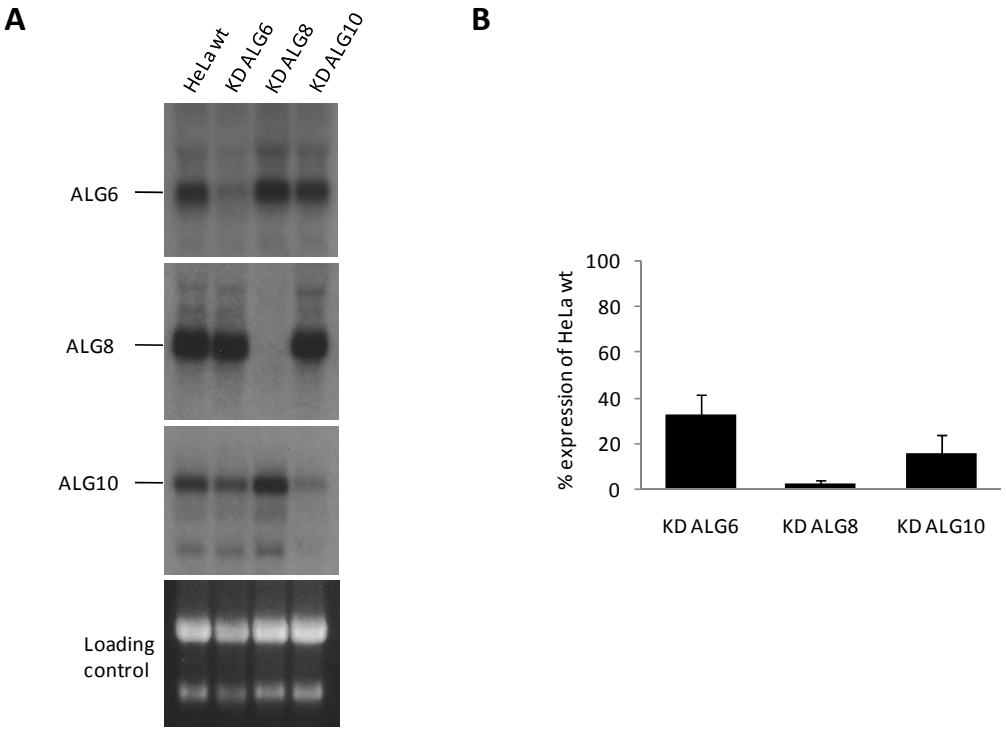

Figure 2 ER glucosyltransferase mRNA levels in knockdown HeLa cells.

A. The decreased mRNA levels were confirmed by Northern blotting. Total RNA from wildtype HeLa and all the knockdown cells was hybridized with radioactive probes against the three ER glucosyltransferases. As a loading control the separating gel was stained with ethidium bromide. **B.** The results from the Northern blots were confirmed by quantitative PCR. The experiment was performed four times independently. The percentage of remaining expression compared to wildtype HeLa cells is shown.

We verified the decrease of ALG6, ALG8 and ALG10 glucosyltransferase activity by investigating the LLO profiles of the knockdown HeLa cells. As expected, the LLO profile of wildtype HeLa cells showed a single peak corresponding to the complete LLO DolPP-$GlcNAc_2Man_9Glc_3$ (Fig. 3). Multiple peaks were visible in the LLO profile of ALG6 knockdown cells, with a major peak corresponding to the expected accumulating DolPP-$GlcNAc_2Man_9$. In

the ALG8 knockdown cells, two peaks were detected, which corresponded to DolPP-$GlcNAc_2Man_9Glc_1$ and DolPP-$GlcNAc_2Man_9$ (Fig. 3). This result was expected since it is known that the ER glucosidase II enzyme can cleave the terminal glucose from DolPP-$GlcNAc_2Man_9Glc_1$ (14). In ALG10 knockdown cells, three major peaks of incomplete LLO were detected. These three peaks represented DolPP-$GlcNAc_2Man_9$, DolPP-$GlcNAc_2Man_9Glc_1$ and the expected DolPP-$GlcNAc_2Man_9Glc_2$ structure. Only the DolPP-$GlcNAc_2Man_9Glc_2$ peak remained visible after treatment of ALG10 knockdown cells with the glucosidase inhibitor castanospermine (data not shown), indicating that ER glucosidase II is also able to cleave the middle Glc of the LLO. The accumulation of the expected LLO structures indicated that the knockdown cell lines were deficient for the ALG6, ALG8 and ALG10 activity, respectively.

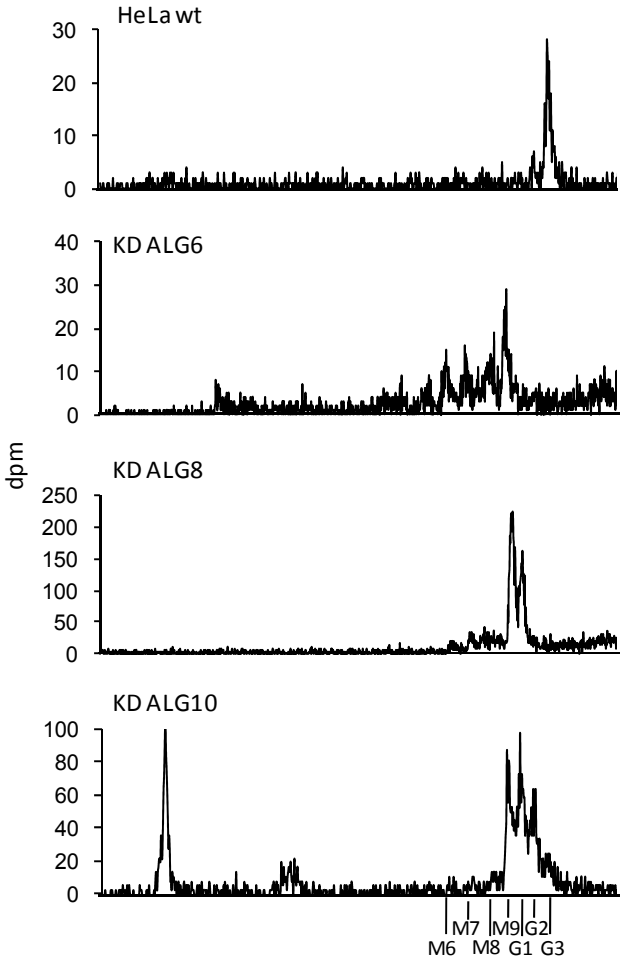

Figure 3 HPLC analysis of the accumulating LLOs in HeLa wildtype and the knockdown cells.

The LLOs were extracted from knockdowns and from HeLa wildtype cells and analyzed by HPLC together with a yeast standard. The accumulating LLOs of the yeast standard are indicated on the x-axis (M6: $GlcNAc_2Man_6$, M7: $GlcNAc_2Man_7$, M8: $GlcNAc_2Man_8$, M9: $GlcNAc_2Man_9$, G1: $GlcNAc_2Man_9Glc_1$, G2: $GlcNAc_2Man_9Glc_2$, G3: $GlcNAc_2Man_9Glc_3$). Radioactivity was measured in disintegration per minute (dpm) and is indicated on the y-axis.

To determine whether the different patterns of LLO accumulation affected the levels of intracellular proteins involved in the folding of glycoproteins, we measured the steady-state levels of ER glucosidase II, protein disulfide isomerase (PDI), ERp57, Bip and calreticulin in knockdown HeLa cells by Western blotting. PDI and ERp57 are thiol-disulfide oxidoreductases that catalyze the formation of disulfide bonds. ERp57 is interacting with calnexin/calreticulin and is especially important for the formation of disulfide bonds in glycoproteins (22, 23). Calreticulin is the soluble component of the protein quality control machinery (24) and BiP is an ER chaperone important for keeping protein regions in a native state before folding (25). These proteins are known to be induced during the unfolded protein response (UPR) in several cell types (26, 27). No difference in ER glucosidase II levels was visible between the cell lines tested (Fig. 4A). Similarly, no changes were detectable for the ER proteins investigated (Fig. 4B). To ascertain whether the proteins investigated can indeed be induced under typical conditions of ER stress (28) we treated the cells with DTT for 24 h. As seen in Fig. 4B, the proteins ERp57, calreticulin and BiP can be induced in wildtype and knockdown HeLa cells by DTT treatment. PDI was only weakly sensitive to DTT. We could not see any difference in the response to DTT between the cells tested.

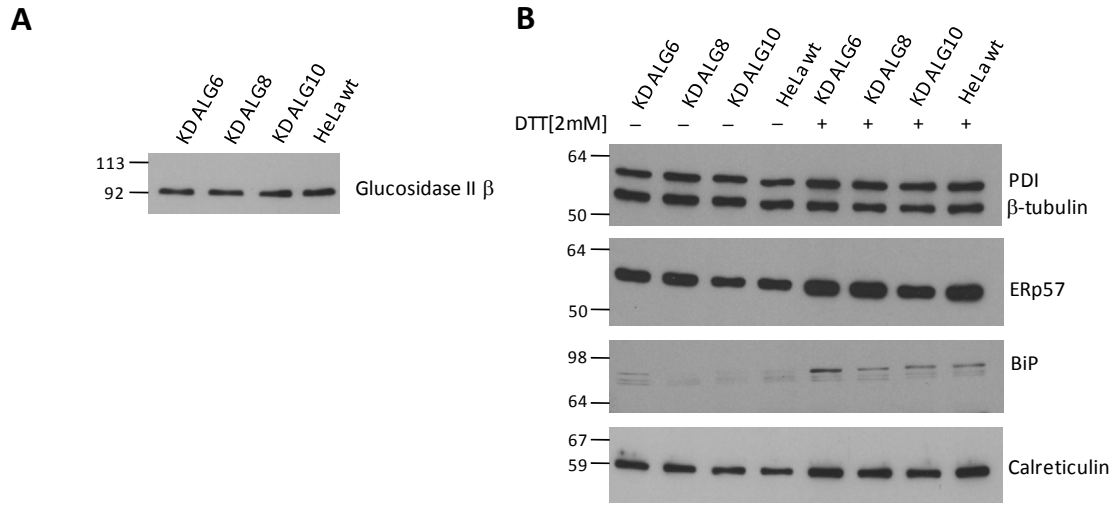

Figure 4 Expression level of glucosidase II and UPR target genes in HeLa wildtype and the knockdown cells.

A. The protein level of glucosidase II in HeLa wildtype and the knockdown cells was analyzed by Western blotting. Molecular weights in kDa are indicated at the left. **B.** HeLa wildtype and the knockdown cells were incubated in presence or absence of 2 mM DTT for 24 h. Western blot analysis of the lysates was performed with antibodies against the four different UPR targets PDI, ERp57, BiP and calreticulin. β-tubulin was used as the loading control. Molecular weights in kDa are indicated at the left.

The levels of ER glucosidase II, PDI, ERp57, Bip and calreticulin were also investigated in primary fibroblasts isolated from ALG6- and ALG8-deficient patients. Western blot analysis showed that the protein levels did not change significantly between wildtype, ALG6- and ALG8-deficient cells (Fig. 5).

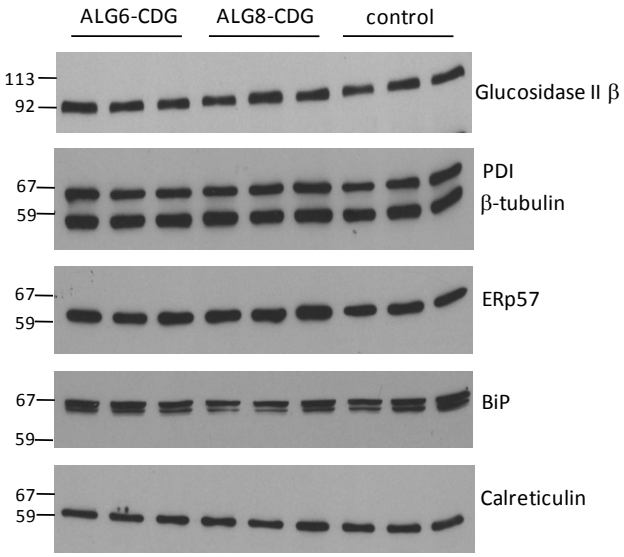

Figure 5 Expression level of glucosidase II and UPR target genes in three ALG6-CDG, three ALG8-CDG and three healthy control fibroblasts.

The protein level of glucosidase II and the four UPR targets were analyzed by Western blotting. Molecular weights in kDa are indicated at the left.

In spite of the apparent lack of ER stress in CDG fibroblasts and ER glucosyltransferase knockdown HeLa cells, we addressed whether the intracellular processing of the endogenous protein cathepsin D was altered. Cathepsin D is a lysosomal protease, which is first translated as an inactive proprotein and catalytically cleaved under way to the lysosome into its activate enzyme form (29). The proprotein found in the ER and Golgi apparatus has a molecular mass of 52 kDa. After a first cleavage in the endosomal compartment, the protein has a mass of 48 kDa. In lysosomes, further cleavage leads to the mature form with two associated peptides of mass 34 and 14 kDa (29). By Western blotting, we could detect the proprotein, the endosomal and the lysosomal form of cathepsin D together with nonspecific bands (Fig. 6). The three specific cathepsin D bands were PNGaseF sensitive whereas the nonspecific bands remained unchanged (data not shown). In HeLa knockdown cells the proprotein form of cathepsin D was hardly visible and most of the protein was processed to the di-peptidic mature form (Fig. 6A). The 34 kDa form did not differ in the intensity between the wildtype and glucosyltransferase knockdown cells, indicating that cathepsin D

processing was not altered due to glucosyltransferase deficiency. Cathepsin D analysis in CDG fibroblasts showed that the proprotein could be detected in all the cells tested at the same level (Fig. 6B). The cleaved endosomal form was detected, too, yet at low level as revealed by the longer exposure of the blot. The intermediary endosomal form was more intense in ALG8 deficient fibroblasts, indicating a possible processing delay in ALG8-CDG (Fig. 6B).

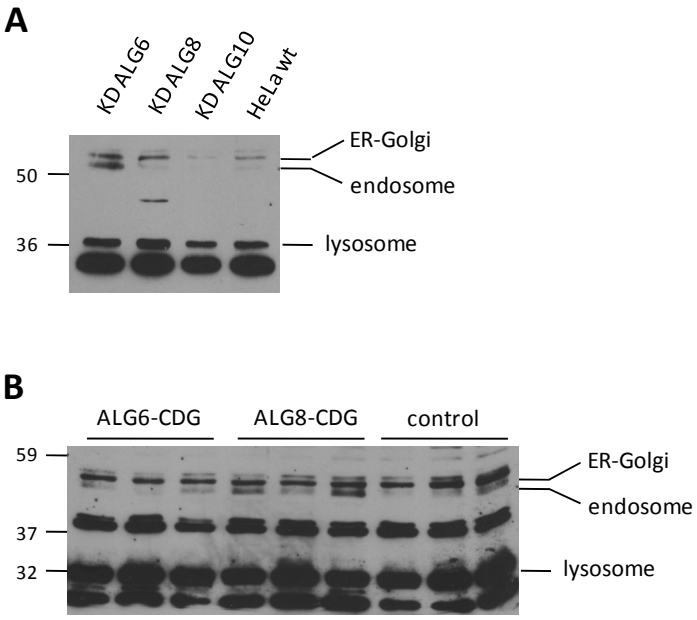

Figure 6 Processing of cathepsin D in knockdown HeLa cells and CDG fibroblasts.

A. Western blot analysis of all the HeLa cells with a cathepsin D antibody. **B.** Western blot analysis of ALG6-CDG, ALG8-CDG and healthy control fibroblasts with a cathepsin D antibody. The processed forms of cathepsin D are indicated at the right and the molecular weights in kDa are indicated at the left.

Defects of LLO biosynthesis lead to unoccupied N-glycosylation sites because of limited substrate availability for the OST complex. Deficient N-glycosylation can impair the folding and secretion of glycoproteins. A model protein often used to investigate secretion and ER-associated protein degradation is α1-antitrypsin, which carries three N-glycans (30). To investigate the glycosylation status of α1-antitrypsin in HeLa knockdown cells, the α1-antitrypsin cDNA was expressed in the HeLa cells by lentivirus infection. After selection of stably expressing HeLa cells, α1-antitrypsin glycosylation was analyzed by Western blotting. Whereas α1-antitrypsin glycosylation was normal in ALG6 and ALG10 knockdown cells, the presence of underglycosylated α1-antitrypsin in ALG8 knockdown cells confirmed the stronger effect of ALG8 deficiency on protein glycosylation (Fig. 7). The identity of the

underglycosylated bands was established by comparison with endogenously produced α1-antitrypsin from hepatoma HepG2 cells.

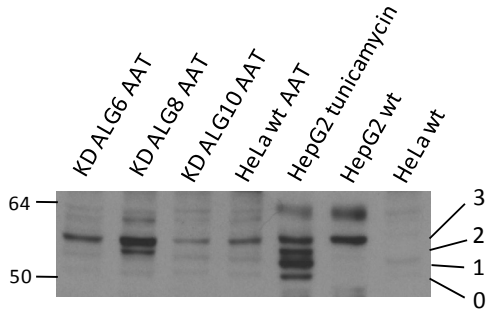

Figure 7 Glycosylation of α1-antitrypsin (AAT) in HeLa wildtype and the knockdown cells.

Western blot analysis of knockdown and HeLa wt cells is shown. HepG2 cells treated with tunicamycin were used to mark the positions of the tri-, di-, mono- and unglycosylated α1-antitrypsin that are indicated at the right. The molecular weights in kDa are indicated at the left.

Overall we can say that the knockdown approach was successful in HeLa cells evaluated by the accumulation of the precursor LLOs. However, we could not identify differences in the cellular responses to the missing glucoses. Even though a slight underglycosylation of the marker protein α1-antitrypsin was detected in the ALG8 deficiency, the folding and secretion efficiency was equal in all the tested cells.

Discussion

The various forms of CDG caused by defective LLO biosynthesis present similar clinical features, yet leading to variable severity. This variability can be explained by the degree of inactivation of the enzyme by the mutation. However, in the case of ALG6-CDG and ALG8-CDG, the former group of patients presents much milder phenotypes than the latter. This might be explained by a loss of additional functions of the ALG8 enzyme than only the transfer of the second glucose or by a loss of functions of its product.

By silencing the three glucosyltransferases in HeLa cells, it was possible to replicate the features of CDG fibroblasts, namely the accumulation of incomplete LLO. The accumulation of the LLO DolPP-$GlcNAc_2Man_9$ and DolPP-$GlcNAc_2Man_9Glc_1$ in ALG8 knockdown cells confirmed that glucosidase II is able to cleave LLO substrates in addition to N-glycans. Similarly, the detection of DolPP-$GlcNAc_2Man_9Glc_2$, DolPP-$GlcNAc_2Man_9Glc_1$ and DolPP-$GlcNAc_2Man_9$ in ALG10 knockdown cells demonstrated that glucosidase II is able to cleave both α1,3-linked Glc form the LLO. The cleavage of the LLOs by glucosidase II suggested that partially glucosylated LLO may have additional roles, possibly regulatory functions, besides representing intermediates in the biosynthetic LLO pathway. The binding of LLO may be especially important for the cleavage of monoglycosylated proteins by glucosidase II, since in this case glucosidase II depends on an extrinsic glycan. The biological importance of a functional glucosidase II activity is highlighted by the disease autosomal dominant polycystic liver disease (ADPLD), which is caused by mutations in the β-subunit of glucosidase II (31). In ADPLD no protein underglycosylation is observed by lectin stainings (31). This observation shows that 50% remaining expression of glucosidase II does not lead to a generalized glycosylation disorder but probably affects a subset of proteins and their folding and secretion (32).

The deficiency of the three ER glucosyltransferase did not appear to affect the efficiency of protein folding significantly considering the lack of an UPR in HeLa knockdown cells and in CDG fibroblasts. Only a minor induction of UPR gene expression was also observed in ALG6-CDG, DPM1-CDG and ALG12-CDG fibroblasts in a previous study (33). The lack of a clear UPR in all these LLO synthesis defects shows that reduced N-glycosylation site occupancy does not disturb protein folding in HeLa cells and fibroblasts. Only the complete loss of N-

glycosylation induced by tunicamycin caused a strong UPR (33) suggesting that already small residual activities of the glycosyltransferases are enough for correct ER quality control.

The processing and glycosylation of the lysosomal protein cathepsin D was not significantly altered in ER glucosyltransferase deficient cells. Whereas the proprotein was detected in the same amount in all fibroblasts tested, the endosomal form was slightly increased in ALG8-CDG fibroblasts. This shows that cathepsin D did not accumulate in the ER but suggests that later processing was delayed in ALG8-deficient fibroblasts. Disturbed targeting of lysosomal proteins was already demonstrated in PMM2-CDG by showing elevated lysosomal enzyme activities in serum (34, 35). Furthermore, in CDG patients with defects in the LLO synthesis pathway abnormal, lysosomal inclusions were found in hepatocytes (36). Furthermore, some symptoms of lysosomal storage disease patients resemble the CDG symptoms suggesting that some CDG symptoms are caused by decreased lysosomal degradation (37).

The efficiency of α1-antitrypsin glycosylation was altered in ALG8 knockdown cells. This observation is compatible with the more severe phenotype of the ALG8 mutant. However, it is important to note that the ALG8 knockdown was the most efficient of the glucosyltransferase knockdowns with mRNA levels down to 3% of normal values. Therefore, we cannot exclude that similar knockdown efficiencies for the *ALG6* and *ALG10* genes would yield similar underglycosylation phenotypes.

The absence of UPR and the very weak trafficking defect of cathepsin D in ER glucosyltransferase knockdown cells suggest that these enzymes do not play a key role in the folding and secretion process of newly synthesized proteins in HeLa cells. Furthermore, knockdown levels of only 50% did not show an accumulation of precursor LLOs (data not shown). This suggests that HeLa cells glycosylate proteins very efficiently also in conditions of decreased LLO availability. However, other cell types like professional secreting cells may be more prone to a protein folding alteration and therefore might show a stronger UPR. Indeed, Dupré et al. showed that α1-antitrypsin of PMM2-CDG patients synthesized in other cell types than hepatocytes, is not underglycosylated (38). Therefore, the knockdown approach should be applied in other cell lines to understand the differences of ALG6 and ALG8 deficiency. We tried to silence the three glucosyltransferases in the hepatoma HepG2 cells as well. However, the knockdown levels achieved in the HepG2 cells were only weak compared to HeLa cells.

In conclusion we propose that low amounts of glucosyltransferase expression are sufficient to hold up normal folding and secretion of proteins in HeLa cells and fibroblasts. Therefore, we think that the ALG8 glucosyltransferase does not play a key role in the folding process of proteins but is an equal member in the LLO synthesis machinery. Further investigations have to be performed in other cell types and in whole organisms to finally conclude that ALG8 has no additional tasks than the transfer of the middle glucose.

Materials and Methods

Materials - Antibodies: PDI, calreticulin, ERp57 (Alexis biochemicals), BiP, glucosidase II (Santa Cruz), α1-antitrypsin, cathepsinD (Abcam), β-tubuline (Sigma).

Cell culture - Primary fibroblasts from skin biopsies, HeLa, HEK293T and HepG2 cells were cultured in DMEM with 4.5 g/l glucose (Invitrogen) and 10% fetal calf serum (BioConcept) at 37°C and 5% CO_2. Tunicamycin (Sigma) was added at 1 μg/ml to HepG2 cells 24 h before collection. DTT (Sigma) was given to the cells in a concentration of 2 mM for 24 h.

Lentiviral transduction - Lentiviral particles containing shRNA sequences against the three ER glucosyltransferases were purchased from Sigma. For the genes *ALG6* (TRCN0000036389, TRCN0000036390, TRCN0000036391, TRCN0000036392) and *ALG8* (TRCN0000034674, TRCN0000034675, TRCN0000034676, TRCN0000034677, TRCN0000034678) several different viruses were transduced. For *ALG10* only one sequence was transduced (TRCN0000034755). 20'000 HeLa cells were plated in 96-well plates one day before giving an MOI of 5 of virus suspension to the cells. 48 h later, selection with 2.5 μg/ml puromycin (Sigma) was started. After selection, every third day new viruses with other target sequences were given to the cells without further selection. Single cell colonies were collected for each knockdown and the knockdown level was measured. The best clones were chosen for further experiments. The cDNA of α1-antitrypsin in pCMV Sport vector was purchased from RZPD (IRATp970A0816D6). The α1-antitrypsin gene was subcloned into pLenti6 vector (Invitrogen). Virus particles were produced in HEK293T cells as described (39). HeLa cells were transduced with the virus containing supernatant of the HEK293T cells and selected with blasticidin (Invitrogen).

RNA isolation and quantitative PCR - Cells were grown to 90% confluence and harvested by trypsinisation. Cell pellets were lysed in Trizol LS reagent (Invitrogen). RNA was extracted according to the suppliers' manual. Reverse transcription was performed with the Omniscript RT kit (Qiagen) with oligo-dT primer. Quantitative PCR was performed with SYBR Green JumpSart Taq ReadyMix (Sigma) on a MX3000P machine (Stratagene). Primers used for the PCR were as follow: ALG6 fw 5'-TGG TGC AGC TTC AAT GTC-3', rv 5-TGA TAG CGC

ACA GCT AAC-3' ; ALG8 fw 5'- GGG TCT ATT CGA TGG AAG AG -3', rv 5'- GTT TGG AGC CCA ATA TGC-3'; ALG10 fw 5'-GGG CCA TTT CTC CCT TTC-3', rv 5'-TTG AGG CAG CCT TGT TTC-3'. Annealing temperature was 60°C.

Northern blot - 5 μg of RNA isolated as described in the previous section was loaded on a 1% agarose-formaldehyde gel and transferred to a Hybond-N nylon membrane (GE Healthcare). Probes were synthesized by PCR from cDNA of healthy control fibroblasts. Primers used for the probe of ALG6 were fw 5'-AGA GTC AGG CAC ATA AGC-3', rv 5'-ACA ACA GAG GGC ATT AGG-3', for the ALG8 probe fw 5'- TAC TCC AGC TCA AGG ACC TTA C-3', rv 5'- CAT GAA CAT GCC ACC CAA AC and for the ALG10 probe fw 5'-CTA CTG TGA GGG CCA TTT CTC C-3', rv 5'-GGA TGT AGG GCC AAG TCA GAA G-3'. PCR products were labelled with $\alpha[^{32}P]$dCTP (Hartmann analytic) with Prime-It II random primer labelling kit (Stratagene) according to the suppliers' manual. Radioactive probes were purified with PCR purification kit (Qiagen). Membranes were hybridized with equal amounts of radioactive probe in QuikHyb hybridization solution (Stratagene) containing 100 μg/ml herring sperm over night at 64°C. Membranes were washed and exposed on films for 7 days.

LLO extraction and HPLC analysis - Labeling, LLO extraction and HPLC analysis was performed as described (39). Briefly, HeLa cells were grown in T150 cell culture flask to 90% confluence. Starvation was performed for 45 min in serum- and glucose-free medium. Labeling was performed for 30 min with 150 μCi of [^{3}H]-mannose. LLOs were isolated by chloroform-methanol-water extraction and released by mild acid hydrolysis. The LLOs were subjected to HPLC together with a yeast standard.

Western blot analysis – Cellular protein lysates were prepared in TBS/1% triton-X100. Equal amounts of lysates were separated in 10% SDS-PAGE under reducing conditions. Transfer was performed on nitrocellulose membrane (Millipore). Signal was detected by ECL kits (Thermo Scientific, GE Healthcare).

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Fibrotic response in fibroblasts from congenital disorders of glycosylation

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Abstract

Congenital disorders of glycosylation (CDG) are characterized by a generalized underglycosylation of proteins. CDG is associated with multiple symptoms like psychomotor retardation, hypotonia, hormonal disorders and coagulopathies. The molecular basis of these symptoms is poorly understood considering the large extent of affected glycoproteins. To identify the cellular responses to protein underglycosylation, the transcriptomes of healthy control and CDG fibroblasts were compared to detect differentially expressed genes. This analysis revealed a strong induction of several genes encoding components of the extracellular matrix, such as collagens, COMP, IGFBP5 and biglycan. The extent of this response was confirmed at the protein level by showing increased production of collagen type-I for example. This fibrotic response of CDG fibroblasts was not paralleled by a differentiation to myofibroblasts and by increased TGFβ signaling. We could show that the addition of recombinant IGFBP5, one of the induced proteins in CDG, to healthy control fibroblasts increased the production of collagen type-I to levels similar to those found in CDG fibroblasts. The fibrotic response identified in CDG fibroblasts may account for the elevated tissue fibrosis, which is often encountered in CDG patients.

Introduction

N-linked glycosylation is a widespread and essential modification of proteins in eukaryotes. Oligosaccharides are first assembled on the lipid-carrier dolichol and then transferred co-translationally to asparagine residues of nascent glycoproteins (1, 2). Loss of N-glycosylation sites on specific proteins leads to various diseases such as metachromatic leukodystrophy (3), thrombophilia (4, 5), and α1-antitrypsin deficiency (6). Defects of lipid-linked oligosaccharide (LLO) biosynthesis cause generalized decreased N-glycosylation sites occupancy due to limited availability of complete LLO in the endoplasmic reticulum (ER). By contrast, N-glycosylation sites occupancy is normal in defects of N-glycan processing, which lead to alteration of N-glycan branching and termination. Both forms of N-glycosylation defects belong to the family of congenital disorders of glycosylation (CDG) (7-9). Considering the broad impact of glycosylation on protein functions, CDG are associated with developmental delays, malformations and multiple organ dysfunctions (10). Symptoms like coagulopathies, gastrointestinal bleedings and liver fibrosis are also often seen in CDG patients. The loss of heparan sulfate on enterocytes has been related to the gastroenteritis-induced protein losing enteropathy encountered in some forms of CDG (11). However, the molecular mechanisms underlying most CDG symptoms are largely unclear.

Alterations of N-glycosylation affect multiple signaling pathways by altering the stability of membrane proteins or the signaling ability of membrane receptors (12). For example, the loss of specific N-glycan branches increases the endocytosis of the glucose transporter GLUT2 (13) and of several cytokine receptors (14). Similarly, changes of N-glycan core fucosylation inhibits TGFβ signaling, which leads to decreased production of extracellular matrix (ECM) proteins and to lung emphysema in β1-6 fucosyltransferase (FUT8) deficient mice (15). Increased core fucosylation of N-glycans has also been observed in CDG cases with defects in the LLO synthesis pathway (16, 17). Whereas the cytokine TGFβ is a central regulator of the production of ECM proteins (18), additional proteins, such as factors of the CCN family (19) and the matricellular protein SPARC (20) have been reported to mediate a profibrotic effect, too.

To gain insight into the pathogenesis of the fibrotic stage associated with CDG, we have investigated the gene expression signature of CDG fibroblasts from patients with defects in the LLO synthesis pathway namely in DPM1 dolichylphosphate mannose synthase (21, 22),

ALG6 glucosyltransferase (23) and ALG12 mannosyltransferase (24-26). This study demonstrated an increased expression of several ECM proteins in CDG and pointed to the involvement of insulin-like growth factor-binding protein (IGFBP) 5 in the mediation of this fibrotic response.

Results

To appreciate the range of cellular responses linked to glycosylation disorders, we have analyzed the global gene transcription profile of ALG6-CDG, DPM1-CDG and ALG12-CDG fibroblasts by oligonucleotide array hybridization (27). Genes with similar expression profiles in the different CDG were resolved by hierarchical clustering. This analysis showed that multiple genes encoding components of the extracellular matrix were highly induced in the three forms of CDG (Fig. 1).

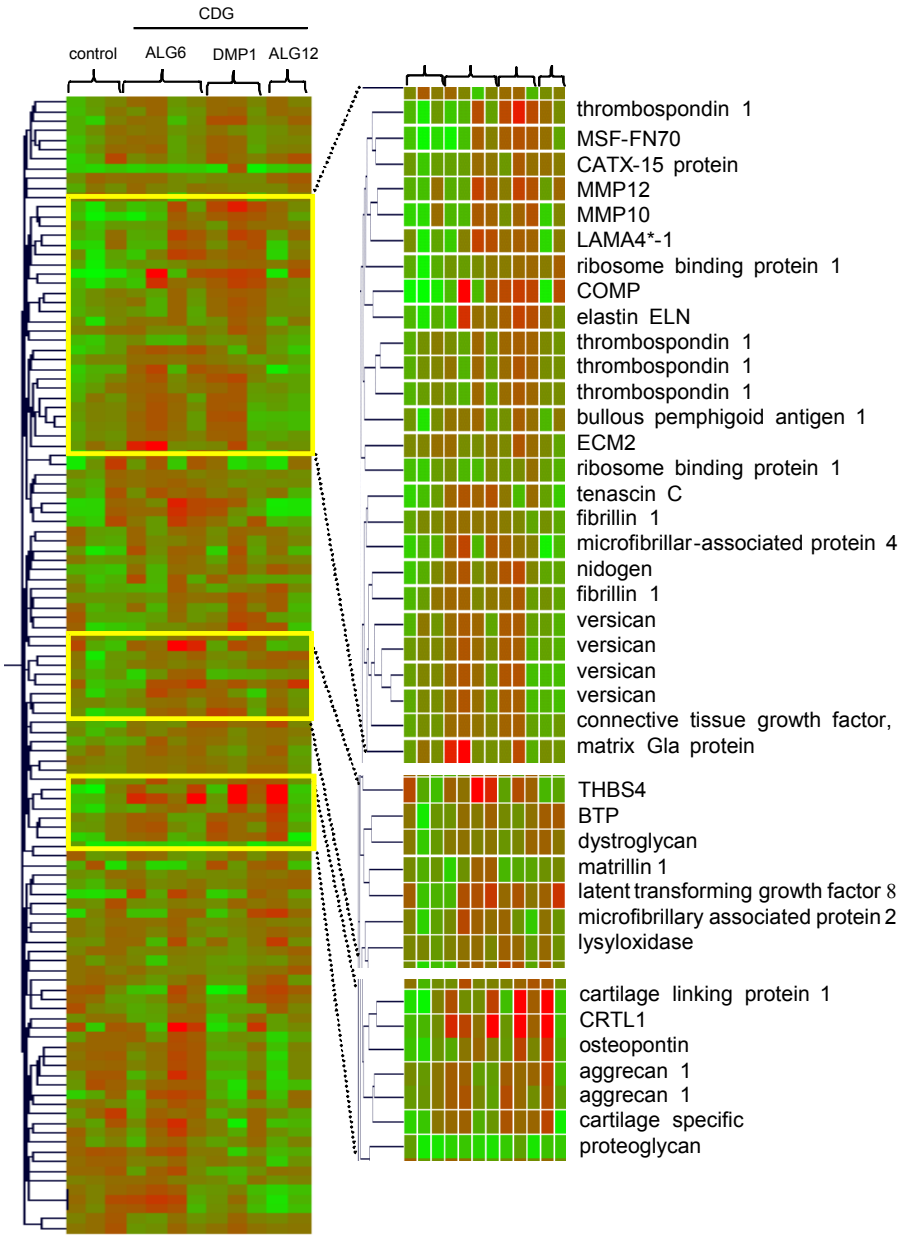

Figure 1 Gene clustering analysis of ECM genes.

The color of a square represents the expression levels relative to the median of all measurements for the gene throughout all the fibroblasts. Red means up-regulated and green means down-regulated in comparison to the median. For some areas with strong differences between controls and CDG patients the gene annotations are shown.

The induced genes comprised collagen genes such as COL1A1, COL4A1, COL4A2, COL5A1, COL11A1 and COL18A1, cartilage oligomeric protein (COMP), biglycan (BGN), hyaluronan and proteoglycan link protein 1 (HAPLN1), fibronectin (FN), thrombospondin-1 (THBS1), tenascin-C (TNC) and nidogen-1 (NID1) in particular (Table 1). Matrix metalloproteinases, like the BMP1 collagenase and the MMP3 proteoglycanase were also among the genes significantly overexpressed in CDG fibroblasts. Another group of ECM genes induced in CDG fibroblasts were four members of the pregnancy specific glycoproteins (PSGs). PSG are closely related to the carcinoembryonic antigen family and are mainly transcribed in the placenta (28), although they are also expressed in other tissues according to the UniGene's EST Profile Viewer (29). Components of the TGFβ pathway were also found among the list of genes induced in CDG. The TGFβ and TGFβ receptor-I mRNAs were slightly elevated in CDG fibroblasts whereas endoglin, a co-receptor for TGFβ (30), was induced between 1.9- and 3.6-fold in CDG fibroblasts (Table 1).

Gene	Symbol	Accession	ALG6-CDG	DPM1-CDG	ALG12-CDG
Collagen type 1, alpha 1	COL1A1	BC036531	3.6	7.2	5.5
Collagen type 4, alpha 1	COL4A1	NM_001845	1.3	2.6	2.3
Collagen type 4, alpha 2	COL4A2	NM_001846	1.3	2.0	2,9
Collagen type 5, alpha 1	COL5A1	NM_000093	1.8	2.1	1.5
Collagen type 11, alpha 1	COL11A1	NM_001854	3.3	10.3	11.9
Collagen type 18, alpha 1	COL18A1	NM_030582	1.5	2.1	2.0
Cartilage oligomeric matrix protein	COMP	NM_000095	18.4	21.1	8.0
Hyaluronan and proteoglycan link protein-1	HAPLN1	NM_001884	5.7	2.1	17.2
Biglycan	BGN	NM_001711	4.4	5.1	2.8
Fibronectin	FN1	NM_212482	1.9	3.8	2.8
Thrombospondin-1	THBS1	AI812030	1.4	4.4	1.8
Tenascin-C	TNC	NM_002160	2.4	1.6	1.0
Nidogen-1	NID1	NM_002508	1.7	2.2	1.0
Tissue factor pathway inhibitor-2	TFPI2	NM_006528	3.2	2.2	0.3
EGF-like repeats and discoidin I-like domains-3	EDIL3	NM_005711	4.4	6.1	7.7

Bone morphogenic protein-1	BMP1	NM_001199	2.1	2.0	2.1
Matrix metalloproteinase-3	MMP3	NM_002422	1.3	3.6	1.7
Pregnancy specific glycoprotein-1	PSG1	NM_006905	6.2	5.0	5.7
Pregnancy specific glycoprotein-4	PSG4	NM_002780	5.3	4.0	5.4
Pregnancy specific glycoprotein-6	PSG6	NM_002782	1.4	1.2	1.4
Pregnancy specific glycoprotein-7	PSG7	NM_002783	6.6	7.3	7.0
Insulin-like growth factor binding protein-5	IGFBP5	R73554	8.1	3.0	1.5
Neuropilin-2	NRP2	NM_201266	1.7	3.0	2.2
Interleukin-8	IL8	NM_000584	2.4	5.5	1.4
Transforming growth factor β	TGFB1	M60316	1.2	1.9	2.0
TGFβ receptor-1	TGFBR1	NM_004612	1.6	1.9	1.3
Endoglin	ENG	NM_000118	1.9	3.6	2.7
Homolog of mothers against decapentaplegic, 7	SMAD7	NM_005904	1.4	1.9	0.8
Wingless-type MMTV integration site family, member 2	WNT2	NM_003391	6.1	2.3	2.5
WNT1-inducible signaling pathway protein 1	WISP1	AB034725	5.5	7.0	5.0
SRY-box 11	SOX11	AB028641	6.3	5.6	5.4

Table 1 mRNA levels of ECM genes in CDG fibroblasts.

The values represent averages of independent measures and indicate the fold-induction compared to the mRNA levels measured in control cells. Fibroblasts from 4 ALG6-CDG, 3 DPM1-CDG and 2 ALG12-CDG cases were investigated. The expression values obtained in the CDG fibroblasts were significantly different from the control values (one-way ANOVA-test, $p < 0.05$).

To validate the up-regulation of ECM components at the protein level, we first measured collagen production in fibroblasts. Using the Sircol dye assay, we detected elevated collagen concentrations in all CDG fibroblasts tested (Fig. 2A). The increased collagen production was confirmed by immunofluorescence analysis of fibroblasts using an antibody to collagen type-I (Fig. 2B).

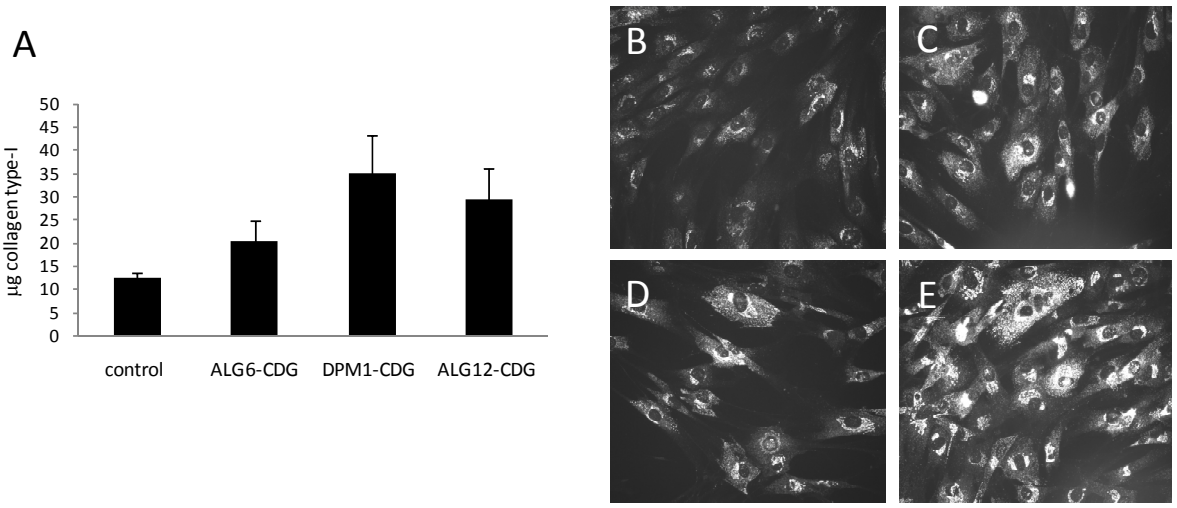

Figure 2 Collagen type-I levels in CDG and healthy control fibroblasts.

A. Total collagen in fibroblasts measured by Sircol assay. **B.** Immunofluorescence staining of collagen type-I in healthy control fibroblasts. **C.** Immunofluorescence staining of collagen type-I in ALG6-CDG fibroblasts. **D.** Immunofluorescence staining of collagen type-I in DPM1-CDG fibroblasts. **E.** Immunofluorescence staining of collagen type-I in ALG12-CDG fibroblasts.

The induction of ECM components in CDG was also confirmed by Western blot analysis of the proteins IGFBP5, COMP, endoglin and PSG1 (Fig. 3). Whereas the production of ECM proteins by fibroblasts is often associated with myofibroblastic differentiation, such a phenotype was not observed in CDG fibroblasts, as assessed by the low level of the myofibroblast marker protein α-smooth muscle actin (31) (data not shown).

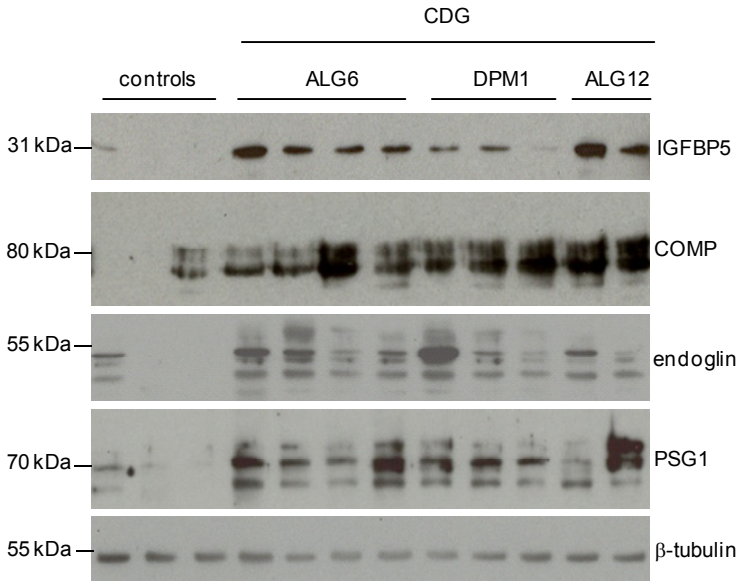

Figure 3 Validation of up-regulated genes on the protein level in control and CDG patient fibroblasts.

Western blot analysis was performed with antibodies against IGFBP5, COMP, endoglin, and PSG1. β-tubulin was used as the loading control. Molecular weights in kDa are indicated at the left.

The cytokine TGFβ is a prominent stimulus of ECM expression (18). To examine whether CDG fibroblasts are more sensitive to TGFβ than control fibroblasts, we treated CDG and control fibroblasts with the profibrotic cytokine TGFβ. The levels of collagen production were strongly increased in all treated cells with the exception of DPM1-CDG fibroblasts (Fig. 4).

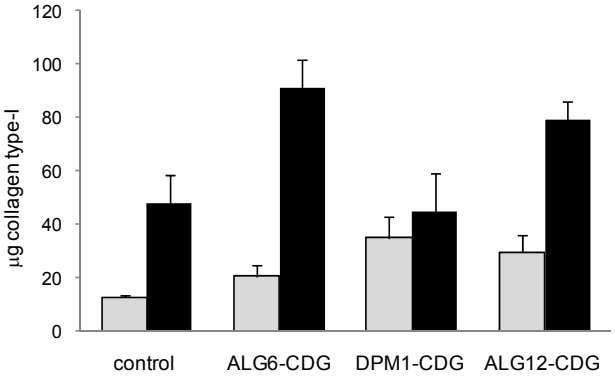

Figure 4 Induction of collagen expression in CDG and healthy control fibroblasts by TGFβ treatment.
Sircol assay was performed from untreated and TGFβ stimulated cells.

We also investigated whether CDG fibroblasts are more sensitive to TGFβ, considering the increased expression of endoglin in those cells. The levels of SMAD2 phosphorylation were measured in control and CDG fibroblasts stimulated with increasing amounts of TGFβ. This titration showed no significant difference of SMAD2 phosphorylation between control and CDG fibroblasts (Fig. 5). Finally, we investigated the activation of the TGFβ pathway by examining the level of SMAD4 nuclear localization in CDG and control fibroblasts. Also this parameter did not differ significantly between the cells examined (data not shown), indicating that the TGFβ pathway did not account for the increased ECM protein expression in CDG fibroblasts.

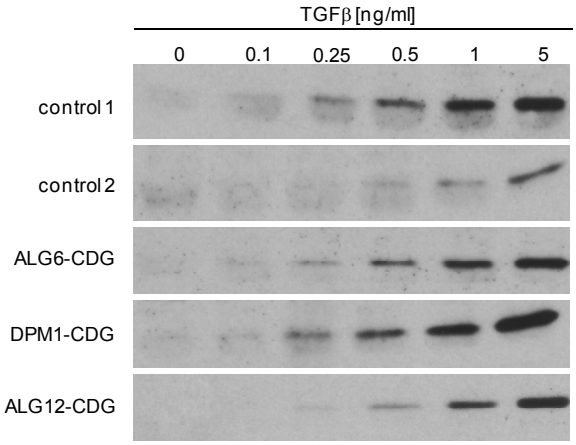

Figure 5 Sensitivity of CDG and healthy control fibroblasts to TGFβ.

Fibroblasts from healthy controls and CDG patients were treated with TGFβ for 30 min. Western blot analysis was performed with an antibody to phosphorylated SMAD2.

The observed increased expression of IGFBP5 in CDG was unexpected. The IGFBP5 protein has been associated with pulmonary fibrosis, where IGFBP5 has been shown to stimulate the production of ECM proteins by activating fibroblasts (32). Therefore, we did test whether the increased IGFBP5 expression in CDG fibroblasts could be related to the increased production of ECM components in these cells. We incubated control fibroblasts with recombinant IGFBP5 at increasing concentrations. The addition of IGFBP5 at 0.25 μg/ml was sufficient to increase the level of collagen production after 2 days, whereas this effect was highest at an IGFBP5 concentration of 1.5 μg/ml (Fig. 6). This effect was similar to that obtained when stimulating fibroblasts with 10 ng/ml of TGFβ.

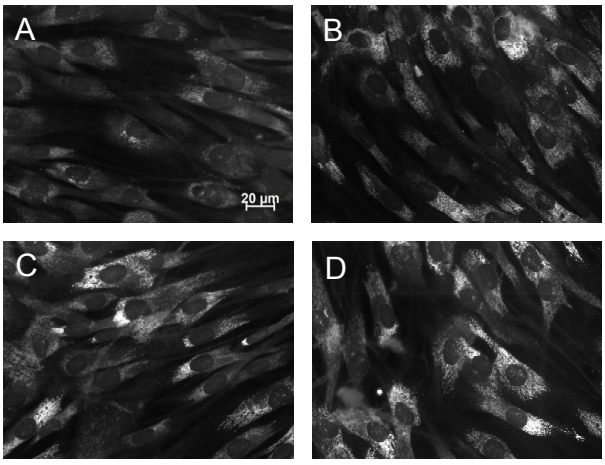

Figure 6 Collagen type-I expression in healthy control fibroblasts after IGFBP5 treatment.

Healthy control cells were treated with 0 **(A),** 0.25 **(B)**, 0.75 **(C)**, 1.5 **(D)** μg/ml of recombinant IGFBP5 and immunofluorescence was performed with a collagen type-I antibody.

Overall we showed that decreased N-glycosylation site occupancy induces the expression of ECM genes. The unchanged TGFβ signaling pathway in control and patient cells pointed to a different molecular mechanism of ECM induction. The fact that it was possible to increase the collagen expression by recombinant IGFBP5 in controls showed that this protein is probably at least partially involved in the induction of ECM gene expression in CDG.

Discussion

N-linked glycosylation is required for the proper function of many glycoproteins. The stability of membrane proteins is depending on the branching of N-glycans and the degree of N-glycosylation site occupancy (12). CDG with defects of LLO synthesis pathway lead to unoccupied N-glycosylation sites. To address the physiological processes and molecular pathways altered by deficient N-glycosylation site occupancy, a global gene transcription array was performed in different forms of CDG. In an earlier study, we could show that the unfolded protein response and amino acid metabolism were induced in CDG fibroblasts (27). In the present work, we have shown that pro-fibrotic genes were also induced in CDG fibroblasts. This observation fits with the frequent observation of tissue fibrosis in CDG patients (33). Identifying the underlying molecular reasons for the up-regulation of ECM genes can give hints for potential therapeutic targets. We first tested the TGFβ signaling pathway as the possible actor. Although some members of the TGFβ signaling pathway were among the induced genes, the whole pathway was not induced in CDG and also the sensitivity of the pathway was not altered. Therefore, a less characterized booster of ECM gene expression came into consideration, namely IGFBP5. Stimulation of control fibroblasts with IGFBP5 indeed showed the same effect of collagen induction as the stimulation with TGFβ. However, the role of IGFBP5 in the induction of ECM genes in CDG fibroblasts was puzzling. The IGFBP5 protein has been previously associated with fibrosis. IGFBP5 is over-expressed in systemic sclerosis fibroblasts and in idiopathic pulmonary fibrosis (32, 34). Adenoviral expression of IGFBP5 in skin of mice induced fibrosis with increased dermal thickness and increased deposition of collagen and fibronectin (35). IGFBP5 is proposed to be a central player in the initiation of fibrosis and in the response to injury (35, 36). However, it is not yet completely understood how IGFBP5 is inducing fibrosis. The family of the IGFBPs is binding with high affinity to the insulin-like growth factors (IGF)-I and –II and therefore regulates the activity of the IFG-axis either positively or negatively (37, 38). The IGF axis is a key regulator of cellular growth, differentiation and apoptosis processes (39). Probably the more relevant mechanism for the ECM induction by IGFBP5 is the ability of IGFBP5 to bind ECM components like collagens, fibronectin and heparan sulfates (40, 41). This binding may protect the ECM proteins from degradation and over-expression of IGFBP5 causes the deposit of big amounts of ECM proteins. The binding of IGFBP5 to ECM proteins

also deprives the pool of free IGFBP5 that can bind IGFs and therefore has an influence on the activity of the IGF pathway (35, 42).

The reasons why protein underglycosylation leads to the up-regulation of IGFBP5 expression are unknown and we can only speculate. Receptor activities are influenced by altered glycosylation. Besides the TGFβ signaling pathway also cytokine receptors are influenced by glycosylation. The overexpression of β1,6-N-acetyl-glucosaminyltransferaseV (MGAT5), which initiates the fourth branch of N-glycans, is leading to a sensitization of the cells to cytokines due to retarded endocytosis of the receptors (14). This sensitization was depending on the composition of the N-glycan, meaning on the MGAT5 expression level, but also on the number of total N-glycans. Therefore, underglycosylation due to a deficient LLO synthesis pathway may alter cytokine response and thus the downstream targets as for example IGFBP5.

The correlation between the underglycosylation of proteins in CDG patients and the observed symptoms are poorly understood. Appreciating and understanding the mechanisms underlying the symptoms represent a first step towards the identification of potential targets for therapeutic approaches. In contrast to developmental defects that cannot be corrected, symptoms like protein-losing enteropathy, coagulation disorders and tissue fibrosis, which create a risk for the condition of living of CDG patients, are reasonable therapeutic targets.

Materials and Methods

Cell culture - Primary fibroblasts from healthy control subjects and from CDG patients were isolated from skin biopsys and were grown in DMEM (Invitrogen) with 4.5 g/l glucose and 10% fetal calf serum.

Collagen determination - Collagen concentration was determined using the Sircol assays (Biocolor) according to the manufacturer instructions. Fibroblasts (2×10^6) were digested overnight in 100 μl of pepsin (0.1 mg/ml, Sigma) in Hanks Balanced Salt Solution, pH 7.0. Collagen was precipitated by the Sircol dye reagent and collected by centrifugation while the unbound dye was removed by adding 0.5 M NaOH. The absorption at 540 nm was measured by spectrophotometry.

Oligonucleotide arrays - Total RNA was isolated from fibroblast cultures and reverse transcribed as described previously (27). cDNA samples were purified by phenol-chloroform extraction and 5 μl were in vitro transcribed in presence of biotin-labeled nucleotides using the High Yield Transcription kit (ENZO Diagnostic USA). Biotin-labeled cRNA samples (15 μg) were fragmented at 95 °C in 40 mM Tris-acetate, 100 mM K-acetate, 30 mM Mg- acetate and mixed in 300 μl of hybridization buffer containing a hybridization control mix, including housekeeping genes (GAPDH, ACO7), spike controls (BIOB, BIOC, CREX, BIODN) and proprietary Affymetrix B2 oligonucleotides, 0.1 mg/ml herring sperm DNA and 0.5 mg/ml acetylated bovine serum albumin in 2-(4-morpholino)-ethane sulfonic acid (MES) buffer, pH 6.7. HG-U133A gene chips (Affymetrix) were incubated with the cRNA samples for 16 h at 45°C, and washed using an Affymetrix Fluidics Station 400. cRNA bound to the oligonucleotide arrays was stained with phycoerythrin-streptavidin and with biotin-labeled anti-streptavidin antibody. The fluorescent signal emitted by the labeled targets was measured using a Gene-Array scanner G2500 (Agilent).

Statistical Analysis - Raw data processing was performed using the Affymetrix Microarray Suite Ver. 5.0 (MAS5) software. After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets using MAS5 software (43). The trimmed mean of each chip was normalized to a target intensity of 500 as recommended by

Affymetrix. Scaling factors and appropriate numbers of present calls (≥ 50%) were calculated by application of a signed-rank call algorithm (44). Statistical parametric methods based on the comparisons between the triplicates of each condition were applied. The Cross-Gene Error Model from the Gene Spring software 5.1. (Silicongenetics, 2003) was applied to filter unreliable genes and unequal variance t-test for two, or ANOVA for more than two groups, were applied to detect differentially expressed genes. The False Discovery Rate was applied (45) to reduce the number of false positive genes.

Immunofluorescence microscopy - Cells were fixed in 3.5 % paraformaldehyde for 10 min and permeabilized with 0.1% saponin in phosphate buffered saline (PBS). Cells were incubated with primary antibodies for 1 h in 0.1% saponin in PBS. After washing twice with PBS, the cells were incubated with Alexa488-conjugated secondary antibody in 0.1% saponin in PBS for 30 min. Immunofluorescence images were taken by an Axiovert 200M microscope (Zeiss) using the Axiovision 3.0 software (Zeiss). The antibodies used were specific to collagen type-I (Sigma, clone COL-1), α-smooth muscle actin (Sigma, clone 1A4), Smad4 (Santa Cruz).

Western blot analysis - Proteins from the postnuclear supernatants of fibroblast cells were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes according to Towbin et al. (46). Membranes were incubated over night with primary antibodies against IGFBP5 (Upstate), COMP (Kamya), PSG1 (BD Biosciences, clone IID10), endoglin (BD Biosciences, clone 35), β-tubulin (Sigma, clone SAP.4G5), pSMAD2 (Cell signalling). After washing in TRIS buffered saline with 0.1% Tween-20, membranes were incubated with a horseradish peroxidase conjugated secondary antibody (Sigma) for 1.5 h and signals were detected by chemiluminescence (Pierce).

IGFBP5 and TGFβ treatment - Fibroblasts from CDG patients and healthy controls were seeded at a density of 10^6 cells in 10 cm plates 24 h prior treatment with TGFβ (10 ng/ml). Treatment with TGFβ was carried out for 36 h prior harvesting for Sircol assay. For the TGFβ sensitivity experiment, $2x10^5$ fibroblasts were seeded in 6-well plates over night. Medium was exchanged with serum free medium for 10 min before adding TGFβ in different concentrations for 30 min to the cells. The reaction was stopped by washing the cells with

ice cold PBS and cells were harvested and lyzed for Western blot analysis. For IGFBP5 treatment, $2x10^5$ healthy control fibroblasts were plated in 6-wells on cover slips. IGFBP5 was given to the medium in the concentrations 0, 0.25, 0.75, and 1.5 μg/ml for 60 h prior to immunofluorescence.

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General discussion

Until recently, glycobiology was merely an exotic research area of biology. One potential reason for this was the lack of reported glycan related diseases or connections to hot topics like cancer and diabetes. A further impediment to glycan research was probably the enormous complexity of glycan structures. However, the view has changed in the last two decades. The discovery of CDG demonstrated the necessity of glycosylation for life and gave insights into the roles of glycans in biological processes. More and more, other areas of biology have encountered glycobiology. Glycans are essential for an efficient immune response (1-3), and the binding and entrance of pathogens into their host cells is often glycan mediated (4). Many developmental processes rely on protein-glycan interactions (5), and changes in glycosylation patterns are today clearly connected to cancer and diabetes as well as other diseases such as multiple sclerosis (6-9). In the area of cancer specifically, altered glycosylation is under investigation as a tumor marker (10). Today we recognize that research in glycobiology is of high value for the understanding of diverse biological processes and diseases. Moreover, glycans are becoming more and more important in medicine as therapeutic targets or as markers.

With the discovery of CDG, glycobiology received a new tool to investigate the synthesis and function of glycans. For most of the *N*-glycosylation synthesis genes, mutations have been discovered in humans. Comparison of the phenotypes of CDG showed high similarities, especially within the class of *N*-glycosylation disorders. However, the severities of the phenotypes are often rather different. The phenotypical similarities prove the linear pathway of *N*-glycan synthesis and show that only a complete *N*-glycan is compatible with healthy, human life. However, if specific CDG are compared to each other in detail, variations in the phenotypes are recognized that do not fit with the expected linearity of *N*-glycan synthesis. As discussed in this thesis the ALG6- and ALG8-CDG patients present rather different clinical pictures despite the proximity of the two enzymes in the LLO synthesis pathway. Another example of an unexpected difference is the absence of neurological symptoms in PMI-CDG patients but the presence of neurological abnormalities in nearly every other CDG, and especially in the PMM2-CDG (11, 12). The PMM2 enzyme is dependent on the product of the PMI enzyme. Nevertheless, the phenotypes are substantially different. Such observations may give hints of not yet considered and discovered roles of glycans and glycan intermediates and add to the general understanding of glycobiology.

The similarities of the phenotypes and the IEF test are the basis in clinics to diagnose a new CDG patient. However, the defective gene is not always identified in each CDG patient and there exists a pool of patients with untyped CDG. In recent years several of these untyped CDG cases have been attributed to a defect in the trafficking machinery. With the description of the first COG7 patients, a new family of proteins was found that is essential for correct *N*- and *O*-glycosylation. In the last few years, mutations in five different COG subunits were found among untyped CDGs. This discovery shows the importance of a functional COG complex for human health and argues for the investigation of the role of the COG complex in the secretory pathway. It is assumed that the COG complex is a tethering factor for retrograde transport of Golgi-resident proteins (13). However, the mechanism of the tethering process is not yet completely understood. A group of proteins known as GEAR proteins have been identified that are sensitive to the deficiency of a COG subunit (14, 15). Some of the GEAR proteins are glycosylation enzymes, explaining the underglycosylation phenotype. However, the relevance of the reduction of the other GEAR proteins is not yet clear. Through the discovery of this new group of CDG, two different research areas met and profited from each other. Comparison of the phenotypes and the glycosylation status in the CDG patients give hints to the tasks and positions of each COG subunit in the complex.

An altered IEF pattern of serum transferrin alone is not enough to conclusively diagnose CDG. Similar patterns of underglycosylation are observed during alcohol abuse (16). Comparison of alcohol abuse and CDG shows not only similarities in the glycosylation status of transferrin but also in the disease characteristics. The most prominent symptom in alcohol abuse is liver cirrhosis and other tissue fibrosis. Tissue fibrosis is also a frequent symptom in CDG. Indeed, in this thesis it was shown that the transcription of extracellular matrix genes is induced in CDG patient fibroblasts with defects in the LLO synthesis pathway. The observation of a similar phenotypical as well as biochemical presentation suggests that similar mechanisms are involved in the pathology of both diseases. It is possible that high levels of alcohol inhibit glycosyltransferases. This was already shown for a Sia-transferase (ST6GAL1) in humans (17). Investigation of the molecular mechanisms for the phenotypes of one disease can add to our understanding of the others.

Glycosylation defects can arise from sources other than defects in the glycosylation machinery. Glycosylation can also be defective in particular proteins due to a mutation in

their glycosylation sites. Not many diseases are known that are caused by the loss of a glycosylation site. An example is found in metachromatic leukodystrophy (18). The loss of the *N*-glycosylation site of sphingolipid activator protein B (SAP-B) inactivates the protein. SAP-B is a cofactor for the cerebroside sulfate degrading hydrolase arylsulfatase A. Therefore, inactive SAP-B causes the inactivation of arylsulfatase A and thus leads to the accumulation of lipids in the lysosomes (19). The fact that only very few diseases are known that are caused by mutation of a glycosylation site suggests that the loss of a glycan is often not harmful. In contrast, in the last few years it has been recognized that the overglycosylation of proteins due to insertion of an *N*-glycosylation site is much more harmful and can cause diseases. It was more than 20 years ago that the first publication claimed that the introduction of a new *N*-glycosylation site can alter protein function (20). Brennan *et al*. showed that a new *N*-glycosylation site in antithrombin inhibits binding to heparin. Therefore, the patient was experiencing massive lung emboli after a clinical surgery and the administration of heparin. However, no functional complementation was performed during this study. Further publications followed showing the correlation between a gain-of-glycosylation and disease. For example a gain-of-glycosylation mutation in the fibrillin 1 gene was identified in Marfan syndrome (21). It was shown that overglycosylation disturbs the oligomerization of microfibrils, an extracellular matrix component consisting predominantly of fibrillin. Another gain-of-glycosylation mutation in antithrombin inhibited the secretion of antithrombin from cells into the blood. Thus, these patients have a higher risk for thrombosis (22). The first clearly verified gain-of-glycosylation mutation was described in the interferon γ receptor2 (23). Here it was shown that the introduction of a new *N*-glycosylation site by a missense mutation changing a Thr to an Asn was sufficient to abolish signal transduction by interferon γ receptor2. Whether this is due to inhibition of binding of interferon γ to the receptor or to the receptor not being able to oligomerize is not yet known. The described patients presented high susceptibility to mycobacterias. Gain-of-glycosylation mutations in a subunit of the $GABA_A$ receptor and in *SRPX2* have also been recently published (24, 25). A database search of all known mutations affecting proteins that are secreted through the ER and Golgi showed that such gain-of-glycosylation mutations are overrepresented. Therefore, gain-of-glycosylation mutations probably contribute to many more disease states than originally thought (26). This suggests that gain-of-glycosylation

mutations may be much more harmful than the loss of a glycosylation site. In addition to the described gain-of-*N*-glycosylation mutations, perhaps pathogenic gain-of-*O*-glycosylation mutations will be found. However, gain-of-*O*-glycosylation defects are not as obvious to detect and investigate because there is no general consensus sequence for *O*-glycans. The suggested large number of diseases with gain-of-glycosylation mutations calls for a general therapeutic approach. Experiments with the *N*-glycosylation inhibitor tunicamycin in cells from patients with the interferon γ receptor2 deficiency showed an increased sensitivity to interferon γ after treatment (23). This result suggests that inhibitors of glycosylation might be adequate drugs for the treatment of gain-of-glycosylation diseases. However, the application of glycosylation inhibitors in humans is difficult because general underglycosylation of proteins causes severe diseases in human as discussed.

Basic researchers in biology are mainly interested in the molecular description and explanation of glycosylation disorders. My thesis as well was mainly focused on molecular and cellular processes in CDG. However, CDG research is of course also relevant for pediatricians. Often, pediatricians are themselves strongly involved in the collection and generation of data on CDG. The recognition and diagnosis of new CDG patients is important to exclude other, possibly treatable diseases and for therapy of the few cases of MPI-CDG and SLC35C1-CDG. With each description of a new case, the clinical picture becomes more precise and CDG becomes more established in the community. Furthermore, development of therapies is dependent on an understanding of the disease. Therefore, it is essential in the quest for a better understanding of rare diseases such as CDG that basic scientists closely collaborate with clinicians and vice versa. For rare diseases, it is important that research groups are connected across many countries to identify the few new cases, to share knowhow and methods, and to work synergistically. These conditions exist in CDG research and have allowed the characterization of many new glycosylation defects in the last decade. It was this close collaboration between medicine and basic research, and the stimulating, international working environment that I appreciated so much during my thesis.

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Abbreviations

AAT	α1-antitrypsin
ADPLD	autosomal dominant polycystic liver disease
AGP	α1-acid glycoprotein
ApoCIII	apolipoprotein CIII
ATP	adenosine triphosphate
BFA	brefeldin A
CDG	congenital disorders of glycosylation
cDNA	complementary DNA
CHO	Chinese hamster ovary
CMP/CTP	cytidine monophosphate/triphosphate
COG	conserved oligomeric Golgi
DMEM	Dulbecco's modified eagle medium
DNA	desoxyribonucleic acid
DolPP	dolichol pyrophosphate
dpm	disintegration per minute
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDEM	ER degradation enhancing α-mannosidase-like
EPO	erythropoietin
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
Fuc	fucose
fw	forward
GAG	glycosaminoglycans
Gal	galactose
GalNAc	N-acetylgalactosamine
GDP/GTP	guanosine diphosphate/triphosphate
Glc	glucose
GlcA	glucuronic acid

GlcNAc	N-acetylglucosamine
GPI	glycosylphosphatidylinositol
HPLC	high performance liquid chromatography
IdoA	iduronic acid
IEF	isoelectric focusing
IGFBP	insulin-like growth factor binding protein
IQ	intelligence quotient
kDa	kilo Dalton
LLO	lipid-linked oligosaccharide
Man	mannose
MOI	multiplicity of infection
mRNA	messenger RNA
NMP/NDP	nucleotide monophosphate/diphosphate
NLO	N-linked oligosaccharide
OMIM	online mendelian inheritance in man
OST	oligosaccharyltransferase
P	phosphate
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
pI	isoelectric point
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription PCR
rv	reverse
shRNA	short hairpin RNA
Sia	sialic acid
TGF	transforming growth factor
UDP	uridine diphosphate
UPR	unfolded protein response
Xyl	xylose

Curriculum Vitae

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Education

07/2005-10/2009	PhD thesis at the Institute of Physiology, University of Zurich, in the group of Prof. Dr. Thierry Hennet "Cellular responses to Congenital Disorders of Glycosylation"
10/1999-05/2004	Bachelor and master studies in biology at the Swiss Federal Institute of Technology of Zurich (ETH) Master thesis at the Institute of Molecular Oncology at University hospital of Zurich in the group of PD Dr. A. Himmelmann "Targeting *PAX5* expression in neural crest derived tumors"
08/1993-08/1999	High school education at the Gymnasium of Glarus, graduated in mathematics and natural sciences (Typus C)

Publications

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